

PATENT  
Docket No.: 19603/3357 (CRF D-1595G)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE



Applicants : Barany et al.

Serial No. : 09/986,527

Cfm. No. : To be Assigned

Filed : November 9, 2001

For : DETECTION OF NUCLEIC ACID  
SEQUENCE DIFFERENCES USING THE  
LIGASE DETECTION REACTION WITH  
ADDRESSABLE ARRAYS

)  
) Examiner:  
) To Be Assigned

)  
) Art Unit:  
) To Be Assigned

**DECLARATION OF GERALD ZON UNDER 37 CFR § 1.608(b)**

Commissioner of Patents  
Washington, D.C. 20231

Dear Sir:

I, GERALD ZON, pursuant to 37 CFR § 1.608(b), declare:

1. I have been active in the field of nucleic acid synthesis, hybridization, detection and sequencing since prior to 1990, and am familiar with DNA, DNA arrays, DNA analogues and their use in nucleic acid sequence detection. A copy of my Curriculum Vitae is attached hereto at Appendix 1.

2. I am currently an employee of Perkin-Elmer/Applied Biosystems, at a California facility of that company. During the period May to June, 1994, I held the position of Vice-President at Lynx Therapeutics, Inc. It is my understanding that Applied Biosystems has a license interest in the above-captioned patent application.

3. I have reviewed the grant application entitled "New Methods for Cancer Detection", ("Grant Application") submitted to the National Cancer Institute, U.S. Department of Health and Human Services that is attached hereto at Appendix 2. This Grant Application describes five (5) projects, including Project 5, entitled "Design and Synthesis of DNA and PNA Arrays". I have focused on Project 5 of the Grant Application. I recall that I first reviewed this Grant Application in the first half of 1994 in connection with the processing of this Grant

Application to determine whether or not to approve the application. While I do not have a complete independent recall of the grant application originally reviewed by me in 1994, it is my recollection that the Grant Application that is attached at Appendix 2 hereto is that 1994 Grant Application.

4. I participated in the NCI Site Special Review Subcommittee visit on May 31, 1994 to June 2, 1994, for the purpose of meeting with scientists submitting the Grant Application that is attached at Appendix 2 hereto, together with other members of the site review subcommittee. My participation, as well as the participation of Steven P.A. Fodor, Ph.D., then Scientific Director and Chief Technical Officer of Affymetrix, Inc. ("Affymetrix") of California, is confirmed by the July 20-22, 1994, Draft Review Report, attached hereto at Appendix 3.

5. I clearly recall the presence of Dr. Fodor throughout the site visit, and his participation on the review subcommittee. It struck me as unusual at the time, because the conflict of interest rules of the grant application review process preclude participation by subcommittee members who are affiliated with institutions pursuing work substantially related to work that is the subject of the grant application in question. I am not an attorney, this is my general understanding of the conduct proscribed by the conflict of interest rules. It was well known at the time that Dr. Fodor and Affymetrix were involved in the development of DNA arrays for the detection of nucleic acid sequence differences. The pursuit of this technology by Dr. Fodor and Affymetrix was already public knowledge by reason of literature and patent publications by both. I recall that my reaction was in the nature of a thought of "what is he doing here?"

6. I recall a substantial focus during the grant application process and review activities on DNA analogues in connection with Project 5 of the Grant Application. It is my memory that this was a particular focus of the work proposed.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: Jan 3, 2002

Gerald Zon.  
Gerald Zon



**Appendix 1**  
(To Declaration of Gerald Zon under 37 CFR § 1.608(b))



## CURRICULUM VITAE

**NAME:** Gerald Zon

**DATE OF BIRTH:** REDACTED

**PLACE OF BIRTH:** Buffalo, New York

**MARITAL STATUS:** Married (Victoria L. Boyd) November 23, 1987; no children

### SUMMARY OF PROFESSIONAL EXPERIENCE AND RESEARCH INTERESTS:

1992- Lynx Therapeutics, Incorporated, Vice President of Medicinal chemistry; responsible for strategic and tactical planning of business-related scientific matters, identifying and initiating research collaborations, interim managing of Quality Control/Regulatory Affairs and general guidance of chemical/analytical work by Lynx R&D/Manufacturing groups.

Specific, noteworthy experience includes the following:

- Directed activities and wrote major sections for U.S. Food and Drug Administration (FDA) Drug Master Files (DMFs) and Phase I Investigational New Drug (IND) applications related to 3 different antisense phosphorothioate oligodeoxynucleotides (S-ODNs) for clinical studies of acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), and coronary restenosis following balloon angioplasty.
- Co-designed and scientifically coordinated a South American Phase I study and a European Phase I/II study both involving an antisense S-ODN for coronary restenosis following balloon angioplasty.
- Co-designed and scientifically coordinated a proof-of-concept study of bioerodeable microspheres for controlled release of antisense S-ODNs *in vivo* to achieve enhanced efficacy and lowered toxicity.

- Successfully pursued 2 separate NIH-sponsored Small Business Innovative Research (SBIR) grants in the areas of antisense and antigene applications of S-ODN technology, the former dealing with the first-ever chemical synthesis of stereopure S-ODNs, and the latter dealing with covalently-attached, triplex-specific intercalators for enhanced potency.

1988-92 Applied Biosystems, Inc., Manager, Therapeutics Group and Head of Quality Control/Regulatory Affairs; responsible for conceiving or identifying, and proposing substantial new business opportunities involving nucleic acid-based drugs as a new venture directed at evolving to a separate business unit within the pharmaceutical industry. Development of core technology (improved raw materials manufacture, synthesis/purification, scale-up chemistry/engineering, and new analytical methods) in conjunction with collaborative research on phosphorothioate pharmacokinetics and specific inhibition of gene expression as potential antiviral and anticancer drugs.

Specific, noteworthy experience includes the following:

- Conceived, developed and published the first method for <sup>35</sup>S-labeling of S-ODNs to enable various antisense-related studies.
- Scientifically coordinated the first-ever pharmacokinetic studies of <sup>35</sup>S-labeled antisense S-ODNs in mice, rats, and monkeys as a prelude for the first IND filing involving continuous, high-dose systemic infusion of an antisense S-ODN in humans.
- By invite, wrote the first review article dealing with progress toward antisense S-ODN treatment of cancer (see attachment).
- By invite, co-authored the first comprehensive review of large-scale synthesis and quality control of antisense S-ODNs, including an unprecedented conceptual comparison of solid-phase vs. solution methodologies (see attachment).

1987-88 Applied Biosystems, Inc., Manager Nucleic Acids Chemistry R & D; responsible for all aspects of nucleic acid chemistry as related to synthesis and sequencing, new applications, new

products, transfer of technology to manufacturing, and interfacing with marketing and sales functions.

Specific, noteworthy experience includes the following:

- Conceived and implemented ABI's world-wide commercialization of automated research-scale antisense S-ODN synthesis and HPLC purification protocols to enable discovery and development of these compounds.
- Co-authored methodology for an automated system useful for synthesis and purification of ODNs utilizing certain high-crosslinked polystyrene matrices.

1984-86 FDA, Chief, Molecular Pharmacology Laboratory; established, staffed and directed this laboratory as an agency priority program to bridge classical drugs and biological products; began basic research in automated synthesis of DNA and analogues thereof for physicochemical and biological studies; established the first DNA synthesis and purification core facility at NIH and within the FDA, which served as a model and technical resource for other such facilities in these federal agencies, as well as USDA, USAMRID, USUS, and academic/private institutions.

Specific, noteworthy experience includes the following:

- Conceived, executed, and published novel methodology for automated synthesis of S-ODNs (see attachment).
- Participated in the design and execution of early antisense S-ODN experiments that utilized a transiently transfected "target"/"reporter" system.
- Participated in the design and execution of early antisense S-ODN studies in cells acutely or chronically infected with HIV-1.

1979-83 FDA, Visiting Chemist (on sabbatical leave and then hired with full-tenure in 1981 by Secretary Department of Health and Human Services); conceived and directed research in polysaccharide vaccine structure determination by chemical and NMR methods; devised new procedures for polysaccharide

endgroup-analysis and coupling these antigenic bacterial products to modified proteins as improved vaccine conjugates.

- 1978-79 Principal Investigator (part time) at Andrulis Research Corporation; established and directed U.S. Army-funded contract research in the design of fabric-attached catalysts for hydrolytic protection from organophosphorus chemical warfare agents.
- 1975-81 Principal Investigator (part time) at Mid-Atlantic Research Institute; established and directed NCI-funded research program in organosilicon chemistry aimed at lipophilic prodrug analogues of anticancer alkylating agents.
- 1973-81 Professorship at The Catholic University of America with pre- and postdoctoral students; directed funded research in novel organosilicon chemistry directed at new reactive intermediates, and organophosphorus chemistry aimed at mechanistic studies of cyclophosphamide (Cytosan®) and the design of improved anticancer analogues.

#### **EDUCATION:**

- 1963 Cardinal Dougherty High School (Valedictorian) Buffalo, NY
- 1967 B.S. Chemistry (Deans Honor List), Canisius College, Buffalo, NY
- 1971 Ph.D., Organic Chemistry, Princeton University, NJ  
Thesis: "I. Hexachlorodisilane as a Reducing Agent II. Synthesis and Pyramidal Stability of Substituted Phosphindoles and Dibenzophospholes" (with Professor Kurt Mislow)
- 1971-73 Postdoctoral, Organic Chemistry, Ohio State University, Columbus OH  
Topic: "Stereochemistry and Mechanisms of Silver Ion-Catalyzed Reactions of Strained Polycyclic Hydrocarbons" (with Professor Leo A. Paquette)

**PUBLICATIONS FOR 1967-1997:** Over 235 (see attached bibliography)

## INVITED REVIEWS AND BOOK CHAPTERS:

1. Zon, G. and Mislow, K. "Studies in Phosphorous Stereochemistry." *Forsch. Chem. Forsch.* **19**, 16, 1971.
2. Zon, G. "NMR Studies of Drug Metabolism and Mechanism of Action." *Magnetic Resonance in Biology*, **1**, 110-170, 1980.
3. Zon, G. "A Review of Cyclophosphamide Analogues," *Progress in Medicinal Chemistry*, Vol 19, Ellis, G.P. and West, G.B. Eds. Elsevier Biomedical, Amsterdam, 1982, pp. 205-246.
4. Egan, W.M., Tsui, F.P., and Zon, G. "Structural Studies of the *Haemophilus Influenzae* Capsular Polysaccharides." *Haemophilus Influenzae*, Sell, S.H. and Wright, R.F. Eds. Elsevier Science Publishing Co., New York, 1982, pp.185-196.
5. Zon, G. and Thompson, J.A. "Review of High-Performance Liquid Chromatography in Nucleic Acids Research. II. Isolation, Purification, and Analysis of Oligodeoxyribonucleotides." *BioChromatogr.*, **1**, 22-23, 1986.
6. Zon, G. "Oligonucleotide Analogues as Potential Chemotherapeutic Agents." *Pharm. Res.*, **5**, 539-549, 1988.
7. Zon, G. "Pharmaceutical Considerations." In *Oligodeoxynucleotides Antisense Inhibitors of Gene Expression*. Cohen, J.S. Ed., CRC Press, 1989, pp. 233-247.
8. Zon, G. "Purification of Synthetic Oligodeoxyribonucleotides." In *High Performance Liquid Chromatography in Biotechnology*, Hancock, W.S. Ed., John Wiley and Sons, 1990, pp. 301-398.
9. Zon, G. and Stec, W.J. "Phosphorothioate Oligonucleotides." In *Oligonucleotides and Analogues: A Practical Approach*, Eckstein, F. Ed., IRL Press, 1991, pp.87-108.
10. Zon, G. and Geiser, T.G. "Phosphorothioate Oligonucleotides: Chemistry, Purification, Analysis, Scale-Up and Future Directions," *Anticancer Drug Design*, **6**, 529-568, 1991.

11. Zon, G. "Phosphorothioate DNA as Investigational New Drugs: Rationale and Production." In *Biotechnology International 1992*, Century Press, 1991, pp. 119-126.
12. Zon, G. "Oligonucleotide Phosphorothioates." In *Protocols for Oligonucleotides and Analogs*, Agrawal, S. Ed., Humana Press, 1993, pp. 165-189.
13. Zon, G. "History of Antisense Discovery." In *Antisense Research and Applications*, Crooke, S.T. and Lebleu, B. Eds., CRC Press, in press.
14. Zon G. "Antisense: Progress Toward Gene-Directed Cancer Therapy" *Encyclopedia of Cancer*, Bertino, J.R. Ed.-in-Chief, Academic Press, 1997, pp. 82-99.

#### **HONORS:**

American Institute of Chemist Award, 1967  
Dow Corning Unrestricted Research Award, 1977-1979  
District of Columbia American Institute of Chemists Award as Outstanding  
Young Teacher, 1978  
Public Health Service Special Recognition Award, 1985

#### **COMMITTEES:**

Science Planning Committee, International Union of Pure and Applied Chemistry, Conference on Phosphorus Chemistry, 1981.

FDA Office of Biologics Research and Review Promotion/Tenure Review Panel, 1985-1987.

Advisory Committee, SUNY-Roswell Park Memorial Institute, Graduate Program in Chemistry/Medical Chemistry, 1985.

NIAID/NIH Study Section of National Cooperative Drug Discovery Groups in AIDS Research, May 1987.

Bioorganic & Natural Products NIH Study Section, February, 1990.

NIGMS/NIH Program Application Review Committee and Site Visit, August, 1990.

NIH SBIR Study Section, 1991

NIH Special Study Sections, 1995 and 1996

**EDITORIAL BOARDS (currently active):**

Nucleic Acids Research

Analytical Biochemistry

Antisense & Nucleic Acid Drug Development



Gerald Zon

To: Gerald Zon/FOS/PEC@PEC

01/03/2002 02:11 PM

cc:

Subject: Jan 3, 2002 ADDENDUM TO CV

June 2001 - present   Applied Biosystems, Senior Director, New & Other Platforms R&D

January 1999 - June 2001   Applied Biosystems, Director R&D, Genetic Analysis Business Unit

June 1997 - December 1998   Inex Pharmaceuticals U.S.A., Vice President, Medicinal Chemistry & Regulatory Affairs

June 1997 (approx.)   Identified and co-managed sale/transfer of all Lynx Therapeutics oligonucleotide technology & assets to Inex Pharmaceuticals  
(Vancouver B.C.) to establish U.S. facility in Hayward CA

## **Appendix 2**

(To Declaration of Gerald Zon under 37 CFR § 1.608(b))

DEPARTMENT OF HEALTH AND HUMAN SERVICES PROGRAM PUBLIC HEALTH SERVICE PROJECT GRANT APPLICATION Follow instructions carefully. Type in the unshaded areas only. Type density must be 10 c.p.i.		LEAVE BLANK FOR PHS USE ONLY.	
Type	Activity	Number	
Review Group	Council/Board (Month, Year)	Formerly	
		Date Received	
1. TITLE OF PROJECT (Do not exceed 56 typewriter spaces.) NEW METHODS FOR CANCER DETECTION			
2a. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OF PROGRAM AND ANNOUNCEMENT Number: Title: CONFIDENTIAL INFORMATION		<input checked="" type="checkbox"/> NO <input type="checkbox"/> YES (If "YES," state number and title)	
2b. TYPE OF GRANT PROGRAM P01		3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR	
3a. NAME (Last, first, middle) BARANY, FRANCIS		3b. DEGREE(S) PH.D.	3c. SOCIAL SECURITY NO. REDACTED
3d. POSITION TITLE ASSOCIATE PROFESSOR		3e. MAILING ADDRESS (Street, city, state, zip code) CORNELL UNIV. MEDICAL COLLEGE 1300 YORK AVENUE NEW YORK, NY 10021	
3f. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT MICROBIOLOGY			
3g. MAJOR SUBDIVISION CORNELL UNIV. MEDICAL COLLEGE			
3h. TELEPHONE AND FAX (Area code, number and extension) TEL: 212 746-6509 FAX: 212 746-8587		BITNET/INTERNET ADDRESS BARANY@CUMC.CORNELL.EDU	
4. HUMAN SUBJECTS If "Yes," exemption no. or IRB approval date 4b. Assurance of compliance no.		5. VERTEBRATE ANIMALS If "Yes," IACUC approval date 5b. Animal welfare assurance no.	
<input checked="" type="checkbox"/> 4a. NO <input type="checkbox"/> YES		<input checked="" type="checkbox"/> 5a. NO <input type="checkbox"/> YES	
6. DATES OF ENTIRE PROPOSED PROJECT PERIOD From (MMDDYY) Through (MMDDYY) 120194 113099		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD 7a. Direct Costs (\$) 7b. Total Costs (\$) \$971,041 \$1,224,862	
		8. COSTS REQUESTED FOR ENTIRE PROPOSED PROJECT PERIOD 8a. Direct Costs (\$) 8b. Total Costs (\$) \$5,295,869 \$6,995,293	
9. PERFORMANCE SITES (Organizations and addresses) CORNELL UNIV. MEDICAL COLLEGE 1300 YORK AVENUE NEW YORK, NY 10021		10. INVENTIONS AND PATENTS (Competing continuation application only) <input type="checkbox"/> NO <input type="checkbox"/> YES If "YES," Previously reported <input type="checkbox"/> Not previously reported	
		11. NAME OF APPLICANT ORGANIZATION CORNELL UNIVERSITY MEDICAL COLLEGE ADDRESS 1300 YORK AVENUE NEW YORK, NY 10021	
12. TYPE OF ORGANIZATION <input type="checkbox"/> Public: Specify <input type="checkbox"/> Federal <input type="checkbox"/> State <input type="checkbox"/> Local <input checked="" type="checkbox"/> Private Nonprofit <input type="checkbox"/> Forprofit (General) <input type="checkbox"/> Forprofit (Small Business)		13. ENTITY IDENTIFICATION NUMBER 1131623978A1 Congressional District 14	
15. NAME OF ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE PHILIP V. GIUCA TELEPHONE 212 746-6036 FAX 212 746-8745 TITLE SENIOR ASSOCIATE DEAN ADDRESS CORNELL UNIV. MEDICAL COLLEGE 1300 YORK AVENUE NEW YORK, NY 10021		14. BIOMEDICAL RESEARCH SUPPORT GRANT CREDIT Code: 01 Identification: SCHOOL OF MEDICINE	
		16. NAME OF OFFICIAL SIGNING FOR APPLICANT ORGANIZATION GREGORY W. SISKIND TELEPHONE 212 746-6020 FAX 212 746-8745 TITLE ASSOCIATE DEAN ADDRESS CORNELL UNIV. MEDICAL COLLEGE 1300 YORK AVENUE NEW YORK, NY 10021	
BITNET/INTERNET ADDRESS QMCUMC.MAIL.CORNELL.EDU		BITNET/INTERNET ADDRESS QMCUMC.MAIL.CORNELL.EDU	
17. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application. Willful provision of false information is a criminal offense (U.S. Code, Title 18, Section 1001). I am aware that any false, fictitious, or fraudulent statement may, in addition to other remedies available to the Government, subject me to civil penalties under the Program Fraud Civil Remedies Act of 1986 (45 CFR 79).		SIGNATURE OF PERSON NAMED IN 3a. (In ink. "Per" signature not acceptable.) Dr. F. Barany	
		DATE 2/4/94	
18. CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true and complete to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as the result of this application. A willfully false certification is a criminal offense (U.S. Code, Title 18, Section 1001). I am aware that any false, fictitious, or fraudulent statement may, in addition to other remedies available to the Government, subject me to civil penalties under the Program Fraud Civil Remedies Act of 1986 (45 CFR 79).		SIGNATURE OF PERSON NAMED IN 16. (In ink. "Per" signature not acceptable.) Gregory W. Siskind	
		DATE 2/4/94	

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. DO NOT EXCEED THE SPACE PROVIDED.

The long range objective of this proposal is to develop sensitive and specific approaches to the detection and simultaneous identification of cancer-related, genetic alterations. Mutations and genetic aberrations have been implicated, at various steps, in the etiology and biology of tumors. Inherited mutations account for the predisposition to cancer in some families. Somatic mutations in tumor suppressor genes, oncogene amplification and viral DNA sequences have been found in cancers as well. However, the clinical use of these discoveries and research into their clinical significance has been slowed by the laborious processes by which they are detected. To apply these discoveries and explore the interactions of multiple genetic alterations, we urgently need a new technology, which is capable of being automated and has the power to detect any of a vast number of mutations.

In response to the urgent need for new methods of mutation detection, we have assembled a team of investigators whose expertise will be directed toward innovative solutions to this problem. The collaborative nature of the scientific and organizational infrastructure will facilitate the attainment of the projects' specific aims and objectives.

The specific aims of the five projects in this proposal are to: (i) develop a multiplex polymerase chain reaction/ligase detection reaction (PCR/LDR) system for the detection of inherited mutations in germline DNA and somatic mutations in tumors; (ii) develop a ligase detection reaction/ polymerase chain reaction (LDR/PCR) system for detecting gene amplifications and deletions in tumors; (iii) develop a PCR/restriction enzyme/LDR (PCR/RE/LDR) system for detecting and identifying mutations in rare cancer cells at a sensitivity of 1 in  $10^6$  or 1 in  $10^7$  by removing normal DNA sequences and selectively amplifying cancer mutations; (iv) design and synthesize nucleotide analogues for converting specific DNA sequences into restriction endonuclease recognition sites for PCR/RE/LDR mutation detection; (v) engineer a thermostable ligase with greater fidelity to enhance LDR and LCR specificity; (vi) design and synthesis oligonucleotide or peptide nucleic acid (PNA) addressable arrays for the simultaneous detection of multiplex LDR and LCR products; and (vii) explore the ability of these technologies to further our understanding and clinical management of lung, colon, breast and cervical cancers.

PERSONNEL ENGAGED ON PROJECT, INCLUDING CONSULTANTS/COLLABORATORS. Use continuation pages as needed to provide the required information in the format shown below on all individuals participating in the project.

Name	AGGARWAL, Aneel	Degree(s)	Ph.D.	Social Security #	REDACTED
Position Title	Assistant Professor	D.O.B.	REDACTED	Role on Project	Co-investigator
Organization	College of Physicians & Surgeons of Columbia University			Department	Biochem & Biophys
Name	AHNEN, Dennis	Degree(s)	M.D.	Social Security #	REDACTED
Position Title	VA Clinical Investigator /Associate Professor	D.O.B.	REDACTED	Role on Project	Collaborator
Organization	Veterans Affairs Medical Center & Univ. Colorado School Of Medicine			Department	Medicine
Name	BARANY, Francis	Degree(s)	Ph.D.	Social Security #	REDACTED
Position Title	Associate Professor	D.O.B.	REDACTED	Role on Project	Prin. Investig.
Organization	Cornell Univeristy Medical College			Department	Microbiology
Name	BARANY, George	Degree(s)	Ph. D.	Social Security #	REDACTED
Position Title	Professor	D.O.B.	REDACTED	Role on Project	Co-investigator
Organization	University of Minnesota			Department	Chemistry
Name	BATT, Carl	Degree(s)	Ph.D.	Social Security #	REDACTED
Position Title	Associate Professor	D.O.B.	REDACTED	Role on Project	Collaborator
Organization	Cornell University			Department	Food Science
Name	BERGSTROM, Donald	Degree(s)	Ph.D.	Social Security #	REDACTED
Position Title	Professor	D.O.B.	REDACTED	Role on Project	Co-investigator
Organization	Purdue Univ. School of Pharmacy & Pharm. Sciences			Department	Medicinal Chem.
Name	BUNK, Michael	Degree(s)	Ph.D.	Social Security #	REDACTED
Position Title	Director, Research Management	D.O.B.	REDACTED	Role on Project	Administrator
Organization	Strang Cancer Prevention Center			Department	Res. Management

Name	COOK, Ronald	Degree(s) Ph. D.	Social Security # REDACTED
Position Title	President/Chief Technical Officer	D.O.B. REDACTED	Role on Project Collaborator
Organization	Siris Laboratories		Department Chemistry
Name	COTHERN, Melissa	Degree(s) B.S.	Social Security # REDACTED
Position Title	Graduate Student	D.O.B. REDACTED	Role on Project Synthetic Chem.
Organization	Louisiana State University		Department Chemistry
Name	COULL, James	Degree(s) Ph.D.	Social Security # REDACTED
Position Title	Group Manager of Specialty Chemistry	D.O.B. REDACTED	Role on Project Collaborator
Organization	Millipore Corporation		Department Specialty Chemistry
Name	COURVALIN, Patrice	Degree(s) M. D.	Social Security # not applicable
Position Title	Professor, Associate Chairman	D.O.B. REDACTED	Role on Project Collaborator
Organization	Institut Pasteur		Department Bacteriol. & Mycol.
Name	DAY, Darren	Degree(s) Ph.D.	Social Security # REDACTED
Position Title	Research Associate	D.O.B. REDACTED	Role on Project -
Organization	Cornell Univeristy Medical College		Department Microbiology
Name	FISHMAN, Jack	Degree(s) Ph. D.	Social Security # REDACTED
Position Title	Director of Research	D.O.B. REDACTED	Role on Project Collaborator
Organization	Strang Cancer Research Lab		Department Horm. Carcinogens
Name	FRANKLIN, Wilbur	Degree(s) M.D.	Social Security # REDACTED
Position Title	Professor, Director of Tissue Bank	D.O.B. REDACTED	Role on Project Collaborator
Organization	University of Colorado, School of Medicine		Department Pathology
Name	FRIEND, Steven	Degree(s) M.D./Ph.D.	Social Security # REDACTED
Position Title	Assistant Professor	D.O.B. REDACTED	Role on Project Collaborator
Organization	Harvard Medical School, MGH Cancer Center		Department Cell & Dev. Biol.
Name	GELFAND, David	Degree(s) Ph.D.	Social Security # REDACTED
Position Title	Director, Core Technology	D.O.B. REDACTED	Role on Project Collaborator
Organization	Roche Molecular Systems		Department Protein Core Res.
Name	GILES, Aaron	Degree(s) B.S.	Social Security # REDACTED
Position Title	Computer Programmer /Analyst II	D.O.B. REDACTED	Role on Project -
Organization	Cornell University Medical College		Department Acad. Computing
Name	GROSSMAN, Larry	Degree(s) Ph.D.	Social Security # REDACTED
Position Title	Professor, Associate Chairman	D.O.B. REDACTED	Role on Project Collaborator
Organization	Wayne State University, School of Medicine		Department Mol. Biol. & Gen.
Name	HACKETT, Neil	Degree(s) Ph.D.	Social Security # REDACTED
Position Title	Assistant Professor	D.O.B. REDACTED	Role on Project Co-investigator
Organization	Cornell Univeristy Medical College		Department Microbiology
Name	HAMMER, Robert	Degree(s) Ph.D.	Social Security # REDACTED
Position Title	Assistant Professor	D.O.B. REDACTED	Role on Project Co-investigator
Organization	Louisiana State University		Department Chemistry
Name	HERRARA, Vicky	Degree(s) M.D.	Social Security # REDACTED
Position Title	Assistant Professor	D.O.B. REDACTED	Role on Project Collaborator
Organization	Boston University School of Medicine		Department Medicine
Name	HOFFMAN, Eric	Degree(s) Ph.D.	Social Security # REDACTED
Position Title	Assistant Professor	D.O.B. REDACTED	Role on Project Collaborator
Organization	University of Pittsburgh, School of Medicine		Department Mol. Gen. Biochem
Name	KENNEDY, Timothy	Degree(s) M.D.	Social Security # REDACTED
Position Title	Medical Director	D.O.B. REDACTED	Role on Project Collaborator
Organization	Lung Cancer Institut of Colorado		Department Institute
Name	KEW, Olen	Degree(s) Ph.D.	Social Security # REDACTED
Position Title	Chief of Molecular Virology Section	D.O.B. REDACTED	Role on Project Collaborator
Organization	National Center of Infectious Diseases, CDC		Department Viral Division
Name	KOLLER, Antje	Degree(s) B.A. equiv.	Social Security # REDACTED
Position Title	Technician	D.O.B. REDACTED	Role on Project -
Organization	Cornell Univeristy Medical College		Department Microbiology
Name	KOVACH, John	Degree(s) M.D.	Social Security # REDACTED
Position Title	Professor, Chairman, Director NCI Cancer Ctr.	D.O.B. REDACTED	Role on Project Collaborator
Organization	Mayo Clinic		Department Oncology

Name	LU, Jing	Degree(s)	B.A.	Social Security #	REDACTED
Position Title	Technician	D.O.B.	REDACTED	Role on Project	
Organization	Cornell Univeristy Medical College			Department	Microbiology
Name	LUBIN, Matthew	Degree(s)	M.D.	Social Security #	REDACTED
Position Title	Director of Medical Genetics	D.O.B.	REDACTED	Role on Project	Co-investigator
Organization	Strang Cancer Prevention Center			Department	Medical Genetics
Name	LUO, Jianying	Degree(s)	Ph.D.	Social Security #	REDACTED
Position Title	Research Associate	D.O.B.	REDACTED	Role on Project	
Organization	Cornell Univeristy Medical College			Department	Microbiology
Name	MILLER, Gary	Degree(s)	M.D./Ph.D.	Social Security #	REDACTED
Position Title	Associate Professor, Director, Histo. Pathology	D.O.B.		Role on Project	Collaborator
Organization	Tissue Procurement Core Lad, Univ. of Colorado School of Medicine			Department	Pathology
Name	NORTHROP, Allen	Degree(s)	Ph.D.	Social Security #	REDACTED
Position Title	Principal Engineer / Adjunct Assistant Prof.	D.O.B.	REDACTED	Role on Project	Collaborator
Organization	Lawrence Livermore National Lab / U. C. S. F. Medical Center			Department	Radiology
Name	OSBORNE, Michael	Degree(s)	M.D.	Social Security #	REDACTED
Position Title	Director	D.O.B.	REDACTED	Role on Project	Collaborator
Organization	Strang Cancer Prevention Center			Department	Surgery
Name	PERSING, David	Degree(s)	M.D./Ph.D.	Social Security #	REDACTED
Position Title	Senior Associate Consultant / Assistant Prof.	D.O.B.	REDACTED	Role on Project	Collaborator
Organization	Mayo Foundation			Department	Lab. Med. & Pathol.
Name	PROUDFOOT, Susan	Degree(s)	M.S.H.A.	Social Security #	REDACTED
Position Title	Executive Director	D.O.B.	REDACTED	Role on Project	Collaborator
Organization	Lung Cancer Institute of Colorado			Department	Lung Institute
Name	REZNIKOV, Leonid	Degree(s)	M.D./Ph.D.	Social Security #	REDACTED
Position Title	Research Fellow	D.O.B.	REDACTED	Role on Project	
Organization	The Children's Hospital & Univ. Colorado Health Sciences Center			Department	Pathology
Name	RIGAS, Basil	Degree(s)	M.D.	Social Security #	REDACTED
Position Title	Associate Professor	D.O.B.	REDACTED	Role on Project	Collaborator
Organization	Cornell University Medical College			Department	Microbiol. & Med.
Name	ROBERTS, Richard	Degree(s)	Ph.D.	Social Security #	REDACTED
Position Title	Director of Research	D.O.B.	REDACTED	Role on Project	Collaborator
Organization	New England BioLabs, Inc.			Department	Eucaryotic Biol.
Name	SHILDKRAUT, Ira	Degree(s)	Ph.D.	Social Security #	REDACTED
Position Title	Director of Research	D.O.B.		Role on Project	Collaborator
Organization	New England BioLabs, Inc.			Department	Research & Devel.
Name	SILVERSTEIN, Saul	Degree(s)	Ph.D.	Social Security #	REDACTED
Position Title	Professor	D.O.B.	REDACTED	Role on Project	Collaborator
Organization	Columbia University			Department	Microbiology
Name	SNINSKY, John	Degree(s)	Ph.D.	Social Security #	REDACTED
Position Title	Senior Director of Research	D.O.B.	REDACTED	Role on Project	Collaborator
Organization	Roche Molecular Systems, Inc.			Department	PCR Research
Name	SOBEL, Mark	Degree(s)	M.D./Ph.D.	Social Security #	REDACTED
Position Title	Chief, Molecular Pathology Section	D.O.B.	REDACTED	Role on Project	Collaborator
Organization	National Cancer Institute			Department	Pathology
Name	SOMMER, Steven	Degree(s)	M.D./Ph.D.	Social Security #	REDACTED
Position Title	Associate Professor	D.O.B.	REDACTED	Role on Project	Collaborator
Organization	Mayo Clinic			Department	Biochem, Mol. Biol.
Name	SOUSSE, Thierry	Degree(s)	Ph.D.	Social Security #	Not applicable
Position Title	Professor	D.O.B.	REDACTED	Role on Project	Collaborator
Organization	Institute de Genetique Moleculaire			Department	Molecular Genetics
Name	SWERDLOW, Harold	Degree(s)	Ph.D.	Social Security #	REDACTED
Position Title	Research Associate	D.O.B.	REDACTED	Role on Project	Collaborator
Organization	University of Utah			Department	Human Genetics
Name	VAGNER, Josef	Degree(s)	Ph.D.	Social Security #	REDACTED
Position Title	Postdoctoral Fellow	D.O.B.	REDACTED	Role on Project	
Organization	University of Minnesota			Department	Chemistry

Name	<u>VAGNEROVA, Lydie</u>	Degree(s)	<u>B.S.</u>	Social Security #	<u>Pending</u>
Position Title	<u>Research Technician</u>	D.O.B.	<u>REDACTED</u>	Role on Project	<u></u>
Organization	<u>University of Minnesota</u>			Department	<u>Chemistry</u>
Name	<u>WANG, Guangyi</u>	Degree(s)	<u>Ph.D.</u>	Social Security #	<u>REDACTED</u>
Position Title	<u>Postdoctoral Fellow</u>	D.O.B.	<u>REDACTED</u>	Role on Project	<u></u>
Organization	<u>Purdue University</u>			Department	<u>Medicinal Chem.</u>
Name	<u>WHITE, Perrin</u>	Degree(s)	<u>M.D./Ph.D.</u>	Social Security #	<u>REDACTED</u>
Position Title	<u>Professor</u>	D.O.B.	<u>REDACTED</u>	Role on Project	<u>Collaborator</u>
Organization	<u>Cornell University Medical College</u>			Department	<u>Pediatrics</u>
Name	<u>WILSON, Geoffrey</u>	Degree(s)	<u>Ph.D.</u>	Social Security #	<u>REDACTED</u>
Position Title	<u>Research Group Leader</u>	D.O.B.	<u>REDACTED</u>	Role on Project	<u>Collaborator</u>
Organization	<u>New England Biolabs, Inc.</u>			Department	<u>Research</u>
Name	<u>WILSON, Vincent</u>	Degree(s)	<u>Ph.D.</u>	Social Security #	<u>REDACTED</u>
Position Title	<u>Associate Professor / Director</u>	D.O.B.	<u>REDACTED</u>	Role on Project	<u>Co-investigator</u>
Organization	<u>The Children's Hospital &amp; Univ. of Colorado School Of Medicine</u>			Department	<u>Pathology</u>
Name	<u>WINN-DEEN, Emily</u>	Degree(s)	<u>Ph.D.</u>	Social Security #	<u>REDACTED</u>
Position Title	<u>Staff Scientist</u>	D.O.B.	<u>REDACTED</u>	Role on Project	<u>Collaborator</u>
Organization	<u>Applied Biosystems, Inc.</u>			Department	<u>Research &amp; Devel.</u>
Name	<u>ZHANG, Peiming</u>	Degree(s)	<u>Ph.D.</u>	Social Security #	<u>REDACTED</u>
Position Title	<u>Postdoctoral Research Assistant</u>	D.O.B.	<u>REDACTED</u>	Role on Project	<u></u>
Organization	<u>Purdue University</u>			Department	<u>Medicinal Chem.</u>

TABLE OF PROPORTIONAL EFFORT OF INVESTIAGTORS

Investigator	Project 1	Project 2	Project 3	Project 4	Project 5	Core A	Core B	Core C	Total	Other Support
A. Aggarwal				10%					10%	75%
F. Barany		15%		10%			10%	5%	40%	65%
G. Barany					10%				10%	67%
D. Bergstrom			10%						10%	50%
N. Hackett						20%			20%	50%
R. Hammer			26%						26%	35%
M. Lubin		10%					10%		20%	0%
V. Wilson	20%								20%	50%



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**Project 2. Genetic Markers of Breast and Cervical Cancer**

**Project Leader: Dr. F. Barany**

**Project Co-Leader: Dr. M. Lubin**

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**Project 3. Design and Synthesis of Nucleotide Analogues**

**Project Leader: Dr. D. Bergstrom**

**Project Co-Leader: Dr. R. Hammer**

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**Project 4. Engineering an Improved Thermostable Ligase**

**Project Leader: Dr. F. Barany**

**Project Co-Leader: Dr. A. Aggarwal**

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**Project 5. Design and Synthesis of DNA and PNA Arrays****Project Leader: Dr. G. Barany**

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**Core B. Instrumentation and Mutation Detection****Core Leader: Dr. F. Barany****Core Co-Leader: Dr. M. Lubin**

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**Core C. Administrative Core****Core Leader: Dr. F. Barany****Core Co-Leader: Dr. M. Bunk**

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DD

Principal Investigator/Program Director (Last, first, middle): F. BARANY, Ph.D.

## DETAILED BUDGET FOR INITIAL BUDGET PERIOD

FROM

THROUGH

94/12/01

95/11/30

## DIRECT COSTS ONLY

PERSONNEL (Applicant Organization Only)					DOLLAR AMOUNT REQUESTED (omit cents)		
NAME	ROLE ON PROJECT	TYPE APPT. (months)	% EFFORT ON PROJ.	INST. BASE SALARY	SALARY REQUESTED	FRINGE BENEFITS	TOTALS
F. Barany, Project 2	Principal Investigator				\$64,288	\$20,572	\$84,860
F. Barany, Project 4	Project Leader				\$28,672	\$9,175	\$37,847
N. Hacket, Core A	Core Director				\$53,400	\$17,088	\$70,488
F. Barany, Core B	Core Director				\$45,938	\$14,700	\$60,638
F. Barany, Core C	Core Director				\$17,300	\$5,536	\$22,836
SUBTOTALS					\$209,598	\$67,071	\$276,669
CONSULTANT COSTS							
Outside Collaborators \$1,000							\$1,000
EQUIPMENT (Itemize)							
Core A, Sparc Station \$15,000							
Core B, FluorImager \$80,100							\$95,100
SUPPLIES (Itemize by category)							
Project 2 \$14,500							
Project 4 \$10,500							
Core A \$16,500							
Core B \$11,000							\$52,500
TRAVEL							
Scientific and Program Meetings \$12,400							\$12,400
PATIENT CARE COSTS		INPATIENT					\$0
		OUTPATIENT					\$0
ALTERATIONS AND RENOVATIONS (Itemize by category)							\$0
OTHER EXPENSES (Itemize by category)							
Proj. 2, \$3,000; Proj. 4, \$3000 Cores: A= \$2,500 B= \$5,000 C= \$5,500							\$19,000
SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD							\$456,669
CONSORTIUM/CONTRACTUAL COSTS							
DIRECT COSTS		Project 1	Lung, Colon	Project 4	X-ray Cryst.	TOTAL	\$514,372
INDIRECT COSTS		Project 3	Nucl. Analogue	Project 5	PNA Arrays		
TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD (Item 7a, Face Page)							\$971,041

**BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD  
DIRECT COSTS ONLY**

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED			
			2nd	3rd	4th	5th
<b>PERSONNEL:</b>						
<i>Salary &amp; fringe benefits</i>						
<i>Applicant organization only</i>		\$276,669	\$367,091	\$381,774	\$397,045	\$412,928
<b>CONSULTANT COSTS</b>		\$1,000	\$1,040	\$1,082	\$1,125	\$1,170
<b>EQUIPMENT</b>		\$95,100	\$6,000	\$76,000	\$6,000	\$6,000
<b>SUPPLIES</b>		\$52,500	\$60,600	\$63,024	\$65,545	\$68,166
<b>TRAVEL</b>		\$12,400	\$12,896	\$13,412	\$13,948	\$14,506
<b>PATIENT CARE COSTS</b>	<b>INPATIENT</b>	\$0	\$0	\$0	\$0	\$0
	<b>OUTPATIENT</b>	\$0	\$0	\$0	\$0	\$0
<b>ALTERATIONS AND RENOVATIONS</b>		\$0	\$0	\$0	\$0	\$0
<b>OTHER EXPENSES</b>		\$19,000	\$19,760	\$20,551	\$21,373	\$22,227
<b>SUBTOTAL DIRECT COSTS</b>		\$456,669	\$467,387	\$555,843	\$505,036	\$524,997
<b>CONSORTIUM/ CONTRACTUAL COSTS</b>		\$514,372	\$535,396	\$556,491	\$578,431	\$601,247
<b>TOTAL DIRECT COSTS</b>		\$971,041	\$1,002,783	\$1,112,334	\$1,083,467	\$1,126,244
<b>TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD</b>						<b>\$5,295,869</b>

(Item 8a)-&gt;

**JUSTIFICATION (Use continuation pages if necessary):**

**From Budget for Initial Period:** Describe the specific functions of the personnel, collaborators, and consultants and identify individuals with appointments that are less than full time for a specific period of the year, including VA appointments.

**For All Years:** Explain and justify purchase of major equipment, unusual supplies requests, patient care costs, alterations and renovations, tuition remission, and donor/volunteer costs.

**From Budget for Entire Period:** Identify with an asterisk (\*) on this page and justify any significant increase or decrease in any category over the initial budget period. Describe any change in effort of personnel.

**For Competing Continuation Applications:** Justify any significant increases or decreases in any category over the current level of support.

**Total 5yr budgets for entire Program Project Grant**

**OTHER SUPPORT**

(Use continuation pages if necessary)

FOLLOW INSTRUCTIONS CAREFULLY. Incomplete, inaccurate, or ambiguous information about OTHER SUPPORT could lead to significant delays in the review and/or possible funding of the application. If there are changes in the information after submission, notify the scientific review administrator of the initial review group before the review; if changes occur after the review, notify the appropriate Institute.

Other support is defined as all funds or resources, whether Federal, non-Federal, or institutional, available to the principal investigator/program director (and other key personnel named in the application) in direct support of their research endeavors through research or training grants, cooperative agreements, contracts, fellowships, gifts, prizes, and other means. Key personnel are defined as all individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project.

Reporting requirements are: for each of the key personnel, describe (1) all currently **active** support and (2) all applications and proposals **pending** review or award, whether related to this application or not. If the support is part of a larger project, identify the principal investigator/program director and provide the data for the relevant subproject(s). If an individual has no active or pending support, check "None." Use continuation pages as needed to provide the required information in the **format** as shown below.

Name Aneel K. Aggarwal Active X Pending \_\_\_\_\_ None \_\_\_\_\_

a. Source and identifying no. NIH, 5 R01-GM44006-04 P.I. Aneel K. Aggarwal

Title Recognition and Cleavage of DNA by Restriction Enzymes

b. Your role on project Principal Investigator % Effort 50%

c. Dates and costs of entire project 4/1/90 to 3/31/95 ; \$ 478,432 (years 1-4 actual supp., year 5 recommended)

d. Dates and costs of current year 4/1/93 to 3/31/94 ; \$ 99,284

e. Specific aims of project To determine the structures of restriction endonuclease BamHI with and without DNA. To crystallize other restriction endonucleases.

f. Describe scientific and budgetary overlap No Scientific or budgetary overlap

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

No Adjustments will be necessary

Name Aneel K. Aggarwal Active X Pending \_\_\_\_\_ None \_\_\_\_\_a. Source and identifying no. NIH, 1 R01 GM49327-01 P.I. Aneel K. AggarwalTitle DNA Recognition and Transcription Control by even-skippedb. Your role on project Principal Investigator % Effort 15%c. Dates and costs of entire project 4/1/93 to 3/31/97 ; \$ 411,449 (year 1 actual, years 2-4 recommended)d. Dates and costs of current year 4/1/93 to 3/31/94 ; \$ 124,699e. Specific aims of project 1. To determine the structures of even-skipped homeodomain with A:T rich and G:C rich DNA. ; 2. The structures of "Pap" fragment and intact even-skipped protein.f. Describe scientific and budgetary overlap No Scientific or budgetary overlap

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

No Adjustments will be necessaryName Aneel K. Aggarwal Active X Pending \_\_\_\_\_ None \_\_\_\_\_a. Source and identifying no. Irma T. Hirschl Faculty Award P.I. Aneel K. AggarwalTitle DNA Recognition and Transcription Regulation by Eukaryotic Transcription Factorsb. Your role on project Principal Investigator % Effort Not applicablec. Dates and costs of entire project 1/1/90 to 12/31/94 ; \$ 100,000d. Dates and costs of current year 1/1/93 to 12/31/93 ; \$ 20,000e. Specific aims of project To purify, characterize, and crystallize even-skipped. To purify, characterize, and crystallize the general transcription factors TFIIB, TFIID with activator VP16f. Describe scientific and budgetary overlap No Scientific or budgetary overlap

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

No Adjustments will be necessary

Name Aneel K. Aggarwal Active \_\_\_\_\_ Pending X None \_\_\_\_\_a. Source and identifying no. Amer. Cancer. Soc. NP-75182 (pending review) P.I. Aneel K. AggarwalTitle Molecular mechanisms governing transcriptional activation in eukaryotesb. Your role on project Principal Investigator % Effort 10%c. Dates and costs of entire project 7/1/94 to 6/30/97 ; \$ 212, 854 ( Requested)d. Dates and costs of current year 7/1/94 to 6/30/95 ; \$ 69,484 (Requested)e. Specific aims of project Crystallization and X-ray analysis of general transcription factors TBP and TFIIB  
with activator VP16f. Describe scientific and budgetary overlap No Scientific or budgetary overlap

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

No Adjustments will be necessary



**OTHER SUPPORT**

(Use continuation pages if necessary)

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Reporting requirements are: for each of the key personnel, describe (1) all currently *active* support and (2) all applications and proposals *pending* review or award, whether related to this application or not. If the support is part of a larger project, identify the principal investigator/program director and provide the data for the relevant subproject(s). If an individual has no active or pending support, check "None." Use continuation pages as needed to provide the required information in the *format* as shown below.

Name Francis Barany Active X Pending \_\_\_\_\_ None \_\_\_\_\_

a. Source and identifying no. NIH 5 RO1 GM41337-05 P.I. Francis Barany

Title Mechanism of Restriction Endonuclease Action

b. Your role on project Principal Investigator % Effort 50%

c. Dates and costs of entire project 12/01/88 to 11/30/93 ; \$ 581,274

d. Dates and costs of current year 12/01/92 to 11/30/93; \$ 122,960 (No cost continuation until 07/01/94)

e. Specific aims of project (i) To characterize wild type and mutant *TaqI* endonucleases. (ii) To identify residues involved in DNA sequence recognition by *TaqI* endonuclease and to isolate mutants with altered sequence recognition. (iii) To determine the properties and sequence of an isoschizomer that recognizes DNA with similar structural motifs as *TaqI*.

f. Describe scientific and budgetary overlap No scientific or budgetary overlap.

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

No adjustments will be necessary.

**OTHER SUPPORT**

(Use continuation pages if necessary)

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Reporting requirements are: for each of the key personnel, describe (1) all currently *active* support and (2) all applications and proposals *pending* review or award, whether related to this application or not. If the support is part of a larger project, identify the principal investigator/program director and provide the data for the relevant subproject(s). If an individual has no active or pending support, check "None." Use continuation pages as needed to provide the required information in the *format* as shown below.

Name Francis Barany Active X Pending \_\_\_\_\_ None \_\_\_\_\_

a. Source and identifying no. Hirschl/Monique Weill-Caulier Career Award P.I. Francis Barany

Title Specificity of thermophilic DNA recognition proteins

b. Your role on project Principal Investigator % Effort Not applicable

c. Dates and costs of entire project 01/01/92 to 12/31/96 ; \$ 100,000

d. Dates and costs of current year 01/01/93 to 12/31/94 ; \$ 20,000 to be used for P.I. salary only

e. Specific aims of project The project aims to identify and characterize new thermophilic DNA recognition proteins.

f. Describe scientific and budgetary overlap The award may only be used to provide salary support for the P.I.

It thus allows the P.I. to spend 40% effort on the NIH RO1 grant, although NIH salary support is at 26% .

There is no budgetary overlap.

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

No adjustments will be necessary.

**OTHER SUPPORT**

(Use continuation pages if necessary)

FOLLOW INSTRUCTIONS CAREFULLY. Incomplete, inaccurate, or ambiguous information about OTHER SUPPORT could lead to significant delays in the review and/or possible funding of the application. If there are changes in the information after submission, notify the scientific review administrator of the initial review group before the review; if changes occur after the review, notify the appropriate Institute.

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Reporting requirements are: for each of the key personnel, describe (1) all currently **active** support and (2) all applications and proposals **pending** review or award, whether related to this application or not. If the support is part of a larger project, identify the principal investigator/program director and provide the data for the relevant subproject(s). If an individual has no active or pending support, check "None." Use continuation pages as needed to provide the required information in the **format** as shown below.

Name Francis Barany Active X Pending \_\_\_\_\_ None \_\_\_\_\_

a. Source and identifying no. Applied Biosystems Inc. P.I. Francis Barany

Title Ligation Amplification Technology

b. Your role on project Principal Investigator % Effort 10%

c. Dates and costs of entire project 02/01/92 to 01/31/97 ; \$ 663,100

d. Dates and costs of current year 02/01/93 to 01/31/94 ; \$ 126,500

e. Specific aims of project The project aims to develop LCR technology

f. Describe scientific and budgetary overlap No scientific or budgetary overlap.

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

No adjustments will be necessary.

Please note that this grant pays the full indirect cost at Cornell Medical College at the time it was awarded.

Applied Biosystems Inc. may not influence or direct the research supported by their grant.

**OTHER SUPPORT**

(Use continuation pages if necessary)

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Name Francis Barany Active X Pending \_\_\_\_\_ None \_\_\_\_\_

a. Source and identifying no. Roche Molecular Systems P.I. Francis Barany

Title Thermophilic SSB, Helicase, and Topoisomerase Genes

b. Your role on project Principal Investigator % Effort 5%

c. Dates and costs of entire project 12/01/92 to 11/30/97 ; \$ 325,365

d. Dates and costs of current year 12/01/92 to 11/30/93 ; \$ 56,300

e. Specific aims of project The project aims to isolate, overexpress, and characterize the thermophilic single-stranded DNA binding protein, helicase, and topoisomerase encoded genes.

f. Describe scientific and budgetary overlap No scientific or budgetary overlap.

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

No adjustments will be necessary.

Please note that this grant pays the full indirect cost at Cornell Medical College at the time it was awarded.

Roche Molecular Systems may not influence or direct the research supported by their grant.

**OTHER SUPPORT**

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Name Francis Barany Active \_\_\_\_\_ Pending X None \_\_\_\_\_

a. Source and identifying no. NIH 5 RO1 GM41337-06 P.I. Francis Barany

Title Mechanism of Restriction Endonuclease Action

b. Your role on project Principal Investigator % Effort 40%

c. Dates and costs of entire project 07/01/94-60/30/99 \$1,004,342

d. Dates and costs of current year \_\_\_\_\_

e. Specific aims of project (i) To identify substrate and co-factor determinants of specific binding and cleavage by *TaqI* endonuclease. (ii) To identify amino acid residues involved in DNA sequence recognition by *TaqI* endonuclease and to isolate mutants with altered sequence recognition or catalytic activity. (iii) To determine the sequence ant thermostability of *TaqI* isoschizomers and chimeric endonucleases.

f. Describe scientific and budgetary overlap There is no scientific overlap. Specific aim 3 will help this NCI proposal. There is no budgetary overlap

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

No adjustments will be necessary.

### OTHER SUPPORT

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Name Francis Barany Active \_\_\_\_\_ Pending X None \_\_\_\_\_

a. Source and identifying no. US Army Med. Rsch. and Devel. Command P.I. Barany, F.

Title Multiplex Detection of Point Mutations, Amplifications and Deletions in Breast Cancer

b. Your role on project Principal Investigator % Effort 10%

c. Dates and costs of entire project 09/01/94 to 08/31/98; \$469,978 (P.I's portion;\$305,485)

d. Dates and costs of current year \_\_\_\_\_

e. Specific aims of project To develop new approaches for the simultaneous detection of genetic alterations in breast cancer by developing: i) a multiplex polymerase chain reaction/ligase detection reaction (PCR/LDR) system for detecting point mutations in tumors; ii) a multiplex ligase detection reaction/polymerase chain reaction (LDR/PCR) system to detect gene amplifications and deletions in tumors; and iii) a polymerase chain reaction/restriction endonuclease/ligase detection reaction (PCR/RE/LDR) system to detect cancer cells present at a level of only 1 in 10<sup>6</sup> cells in clinical specimens.

f. Describe scientific and budgetary overlap There will be some scientific and budgetary overlap. This DOD proposal is a pilot version of the more detailed experiments described in Project 2.

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

Some adjustments will be necessary.

**OTHER SUPPORT**  
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Name George Barany Active X Pending \_\_\_\_\_ None \_\_\_\_\_

a. Source and identifying no. NIH GM 43552-04 P.I. George Barany

Title Sulfur-Sulfur Bridging in Solid-Phase Peptide Synthesis

b. Your role on project Principal Investigator % Effort 10% + 2 month summer

c. Dates and costs of entire project 09/01/90 - 08/31/95, \$472,467

d. Dates and costs of current year 09/01/93 - 8/31/94, \$91,574

e. Specific aims of project Building on our twin expertises in mild chemical methods for solid-phase peptide synthesis and in sulfur chemistry, we aim to develop general methods for the creation of one or more sulfur-sulfur bonds in biologically active peptides such as oxytocin, battenecin, apamin and neutrophil defensins.

f. Describe scientific and budgetary overlap None

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

None

**OTHER SUPPORT**

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Name George Barany Active X Pending \_\_\_\_\_ None \_\_\_\_\_

a. Source and identifying no. NIH GM 42722-05 P.I. George Barany

Title Improved Handles for Solid Phase Peptide Synthesis

b. Your role on project Principal Investigator % Effort 10% + 1 month summer

c. Dates and costs of entire project 7/01/93 - 6/30/97, \$576,062

d. Dates and costs of current year 7/01/93 - 6/30/94, \$144,598

e. Specific aims of project The application of several recently worked-out handles to the stepwise segment condensation syntheses of challenging peptide targets, and simultaneous examination of modified and new chemistries that may lead to new handles with even better properties.

f. Describe scientific and budgetary overlap None, although it should be pointed out that some of the advances on GM 42722 will facilitate studies proposed in the present application

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

None



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Name George Barany Active \_\_\_\_\_ Pending X None \_\_\_\_\_

a. Source and identifying no. NIH GM 51628-01 P.I. George Barany

Title Synthetic Studies of Protein Stability and Folding

b. Your role on project Principal Investigator % Effort 10% + 1 month summer

c. Dates and costs of entire project 07/01/94 - 06/30/98, \$649,219

d. Dates and costs of current year 07/01/94 - 06/30/95, \$167,246

e. Specific aims of project The application of state-of-the-art mild chemical methods for peptide synthesis to prepare analogues of bovine pancreatic trypsin inhibitor (BPTI), which are then studied by a series of biophysical techniques in order to learn about structure, dynamics, and folding. Dr. Clare Woodward is an equal co-investigator on this proposal.

f. Describe scientific and budgetary overlap None

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

None

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Name George Barany Active X Pending X None       

a. Source and identifying no. Selectide Corporation, Tucson Arizona P.I. George Barany

Title Novel Beaded Supports for Peptide Library Procedures

b. Your role on project Principal Investigator % Effort 5%

c. Dates and costs of entire project 10/0192 - 09/30/93, \$70,000

d. Dates and costs of current year 10/0192 - 09/30/93, \$70,000 (second year funding is pending)

e. Specific aims of project Academic-industrial collaboration between the P.I. who has experience with development and applications of supports for solid-phase peptide synthesis, and company which holds patents for novel "one bead-one peptide" library process

f. Describe scientific and budgetary overlap None, although it should be pointed out that the "shaving" technology referred to in this proposal was originally developed under the Selectide grant for an unrelated application

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

None

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Name Donald E. Bergstrom Active X Pending \_\_\_\_\_ None \_\_\_\_\_

a. Source and identifying no. NIH, AI26029-07 P.I. Donald E. Bergstrom

Title Design and Synthesis of Oligonucleotide Analogue Antivirals

b. Your role on project Principal Investigator % Effort 10%

c. Dates and costs of entire project 07/01/91 through 06/30/94, \$266,417

d. Dates and costs of current year 07/01/93 through 06/30/94, \$95,870

e. Specific aims of project This project is concerned with the design and synthesis of oligonucleotides modified at T and dG with groups designed to facilitate cleavage of complementary ribonucleic acids. The modified oligonucleotides are to be tested as antiviral agents.

f. Describe scientific and budgetary overlap No scientific or budgetary overlap.

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

No adjustments will be necessary.

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Name Donald E. Bergstrom Active X Pending \_\_\_\_\_ None \_\_\_\_\_

a. Source and identifying no. Walther Cancer Institute P.I. Donald E. Bergstrom

Title "Walther Professor"

b. Your role on project Principal Investigator % Effort 40%

c. Dates and costs of entire project 007/01/89 through 06/30/94, \$500,000

d. Dates and costs of current year 07/01/93 through 06/30/94, \$100,000

e. Specific aims of project To develop anticancer drugs and tools for biochemical investigations on the process of  
oncogenesis.

f. Describe scientific and budgetary overlap The Walther grant provides supplemental funds unavailable through federal  
funding sources. Approximately 50% of the funds are salary for the P.I., and the remainder supports a  
postdoctoral fellow

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

No adjustments will be necessary.

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Name Donald E. Bergstrom Active X Pending \_\_\_\_\_ None \_\_\_\_\_

a. Source and identifying no. NCI, 5-T32-CA09634-04 P.I. William M. Baird, Co-P.I.

Title Training in Drug and Carcinogen-DNA Interactions

b. Your role on project Research Director of one supported graduate student % Effort \_\_\_\_\_

c. Dates and costs of entire project 09/30/90 through 08/31/95, \$923,805

d. Dates and costs of current year 09/30/93 through 08/31/94, \$148,130

e. Specific aims of project \_\_\_\_\_

f. Describe scientific and budgetary overlap Eighty percent of the support for one graduate student is derived from this grant. The project involves the development of oligonucleotides linked to redox active metal complexes to be used for both antiviral and anticancer drug development.

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

No adjustments will be necessary.

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Name Donald E. Bergstrom Active \_\_\_\_\_ Pending X None \_\_\_\_\_

a. Source and identifying no. NIH, 1R01 AI36601-01 P.I. Donald E. Bergstrom

Title Design and Synthesis of Oligonucleotide Analogs

b. Your role on project Principal Investigator % Effort 10%

c. Dates and costs of entire project 07/01/94 through 06/30/98, \$458,609

d. Dates and costs of current year \_\_\_\_\_

e. Specific aims of project This proposal is a competitive renewal of NIH, U01 AI26029-07. The grant has been submitted as an R01 since the primary goal is directed more towards the solution of synthetic and biochemical problems, rather than the development of antiviral agents. The objective continues to be the development of sequence-specific synthetic nucleases.

f. Describe scientific and budgetary overlap No scientific or budgetary overlap.

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

No adjustments will be necessary.

**OTHER SUPPORT**  
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Name Donald E. Bergstrom Active \_\_\_\_\_ Pending X None \_\_\_\_\_

a. Source and identifying no. NIH, STTR 94-1 P.I. Donald E. Bergstrom

Title Development of Universal Nucleic Acid Components

b. Your role on project Subcontract to Integrated Biotechnology Corporation, % Effort 10%  
Carmel, Indiana

c. Dates and costs of entire project 07/01/94 through 06/30/95, \$12,384

d. Dates and costs of current year \_\_\_\_\_

e. Specific aims of project Same as SBIR 93-25

f. Describe scientific and budgetary overlap Same as SBIR 93-25

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

If both SBIR 93-28 and STTR 94-1 are funded, one will not be accepted since they are essentially identical p  
roposals.

**OTHER SUPPORT**

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Name Donald E. Bergstrom Active \_\_\_\_\_ Pending X None \_\_\_\_\_

a. Source and identifying no. NIH, SBIR 93-25 P.I. Donald E. Bergstrom

Title Development of Universal Nucleic Acid Components

b. Your role on project Subcontract to Integrated Biotechnology Corporation, % Effort 10%  
Carmel, Indiana

c. Dates and costs of entire project 07/01/94 through 12/31/94, \$14,098

d. Dates and costs of current year \_\_\_\_\_

e. Specific aims of project This project is concerned with the biochemical comparison of the five nucleoside analogs  
designed to function as "wild cards" for PCR and dideoxy sequencing. Our role will be to synthesize  
the nucleosides for experiments to be conducted by Integrated Biotechnology Corporation.

f. Describe scientific and budgetary overlap There is no budgetary overlap. The scientific overlap is minimal. One of  
the five nucleoside analogs to be synthesized for Integrated Biotechnology Corporation will also be  
synthesized for testing by participants on the current proposal.

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

If both SBIR 93-28 and STTR 94-1 are funded, one will not be accepted since they are essentially identical  
proposals.



**OTHER SUPPORT**

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Name Neil Hackett Active \_\_\_\_\_ Pending X None \_\_\_\_\_

a. Source and identifying no. NIH, RFA AI-93-023, 'Tuberculosis Diagnostics' P.I. Neil R. HACKETT

Title Rapid detection of Mycobacterium drug resistance

b. Your role on project Principal Investigator % Effort 40%

c. Dates and costs of entire project 94/10/01 - 97/09/31, \$600,000

d. Dates and costs of current year 94/10/01 - 95/09/31, \$250,000

e. Specific aims of project The Aims of this Project are:

1. Detect drug resistance mutations in *M. tuberculosis* by multiplex PCR/LDR.

2. Screen 500 *M. tuberculosis* isolates for pattern of drug resistance.

3. Automate PCR/LDR reactions on laboratory robot.

4. Automate DNA extraction from sputum and interface with PCR/LDR method

f. Describe scientific and budgetary overlap No budgetary overlap

The tb proposal applies the simplest method of the cancer detection protocol to a different experimental system. Its focus is on epidemiological studies of tb and on automation. Success in either project would augment the other but there is no overlap of effort or resources.

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

No Adjustments will be necessary

Name Neil Hackett Active X Pending \_\_\_\_\_ None \_\_\_\_\_a. Source and identifying no. NIH, 7-R29-HG00349-05 P.I. Neil HackettTitle Physical mapping an archaeobacterial genomeb. Your role on project Principal Investigator % Effort 50%c. Dates and costs of entire project 88/09/01 - 93/08/31 \$350,000d. Dates and costs of current year 93/09/01 - 94/08/31. Non-funded continuation (\$40,000 carry over)e. Specific aims of project To create a physical map of the genome of *H. halobium* using pulsed field gel electrophoresis. To compare chromosomes of two halobacterial strains.f. Describe scientific and budgetary overlap No Scientific or budgetary overlap

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

No Adjustments will be necessaryName Neil Hackett Active X Pending \_\_\_\_\_ None \_\_\_\_\_a. Source and identifying no. Cornell Scholarship P.I. Neil HackettTitle Protein-DNA interaction in Halobacteriab. Your role on project Principal Investigator % Effort 50%c. Dates and costs of entire project 08/01/90 - 07/31/93, \$200,000d. Dates and costs of current year 08/01/93 - 07/31/94. Non-funded continuation (\$30,000 carry over)e. Specific aims of project To purify the repressor of a phage which infects *Halobacterium* and to assay the interaction with DNA by biochemical and genetic methods.f. Describe scientific and budgetary overlap No Scientific or budgetary overlap

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

No Adjustments will be necessary

Name Neil Hackett Active \_\_\_\_\_ Pending X None \_\_\_\_\_a. Source and identifying no. N.S.F. P.I. Neil HackettTitle Mechanism of DNA binding by a halophilic phage repressor.b. Your role on project P.I. % Effort 40%c. Dates and costs of entire project 06/01/94 - 05/30/97, \$ 300,000d. Dates and costs of current year 06/01/94 - 05/30/95, \$ 100,000e. Specific aims of project To purify a repressor from a phage which infects Halobacterium and determine mechanism by biochemical and genetic methods.f. Describe scientific and budgetary overlap No Scientific or budgetary overlap

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

No Adjustments will be necessaryName Neil Hackett Active \_\_\_\_\_ Pending X None \_\_\_\_\_a. Source and identifying no. Heiser foundation P.I. Neil HackettTitle Regulation of Mycobacterium gene expressionb. Your role on project Principal Investigator % Effort 40%c. Dates and costs of entire project 94/06/01 - 96/05/31, \$40,000d. Dates and costs of current year 94/06/01 - 95/05/31, \$20,000e. Specific aims of project To explore the role a two-component regulatory system in the control of gene expression in Mycobacterium tuberculosis.f. Describe scientific and budgetary overlap No scientific or budgetary overlap.

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

No Adjustments will be necessary

**OTHER SUPPORT**

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Name Robert P. Hammer Active X Pending \_\_\_\_\_ None \_\_\_\_\_

a. Source and identifying no. ACS-PRF #26761-G4 P.I. \_\_\_\_\_

Title New Methods for the Preparation of Methylene Phosphonate Oligonucleotides

b. Your role on project Principal Investigator % Effort \_\_\_\_\_

c. Dates and costs of entire project Jan. 1, 1993 - Aug. 30, 1995; \$20,000

d. Dates and costs of current year Jan. 1, 1993 - Dec. 31, 1994; \$10,000

e. Specific aims of project The Aims of this project are:

1. Synthesis of H-phosphinate mononucleosides starting from natural mononucleosides;

2. Synthesis and biophysical characterization of methylene phosphonate oligonucleotides with direct comparison of properties to analogous DNA or RNA oligomers;

3. Application of methylene phosphonate oligonucleotides as inhibitors of gene expression in vitro and in vivo.

4. Evaluation of methylene phosphonate oligoRNA's as ribozymes.

f. Describe scientific and budgetary overlap. No scientific or budgetary overlap

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

No adjustments will be necessary

**OTHER SUPPORT**

(Use continuation pages if necessary)

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Name Robert P. Hammer Active X Pending        None       

a. Source and identifying no. Louisiana Educational Quality Support Fund 033A P.I.       

Title Homochiral Phosphono-substituted Peptide Analogs

b. Your role on project Principal Investigator % Effort 15%

c. Dates and costs of entire project June 1, 1993 - June 30, 1996; \$105,000

d. Dates and costs of current year June 1, 1993 - June 1, 1994; \$35,000

e. Specific aims of project The Aims of this project are:

1. Experimental identification of potential boron auxiliaries by solution NMR studies of various phosphonite chiral boron compounds;
2. Deprotonation and alkylation of the  $\alpha$ -carbon of the boron-phosphonite complex with evaluation of level of chiral induction by NMR and optical rotation of phosphonite amino acids;
3. Coupling of phosphonite amino acids with stereocontrol at phosphorus;
4. Synthesis of phosphono-substituted peptides using the techniques advanced in Aims 1-3.

f. Describe scientific and budgetary overlap No scientific or budgetary overlap

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

No adjustments are necessary

**OTHER SUPPORT**

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Name Robert P. Hammer Active X Pending        None       

a. Source and identifying no. Louisiana Educational Quality Support Fund 066A P.I. Steven A. Soper

Title Single Lane, Single Dye Sequencing Using NIR Detection

b. Your role on project Co-Principal Investigator % Effort 10%

c. Dates and costs of entire project June 1, 1993 - June 30, 1996; \$139,509

d. Dates and costs of current year June 1, 1993 - June 1, 1994; \$64,559

e. Specific aims of project The Aims of this project are: To develop a single lane DNA sequencing strategy using NIR fluorescence and heavy-atom modified NIR dyes. NIR labeling dyes will be prepared and their photophysics characterized using a variety of fluorescence techniques. These dyes along with ultrasensitive NIR detection, will be used in Sanger dideoxy sequencing using capillary gel electrophoresis. The fluorescence will be excited using a NIR diode laser. Identification of the terminal base will be accomplished by measuring fluorescence lifetimes.

f. Describe scientific and budgetary overlap There is significant scientific and budgetary overlap with NIH and DOE applications with Soper. No significant overlap with the current application.

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

No adjustments necessary

**OTHER SUPPORT**

(Use continuation pages if necessary)

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Name Robert P. Hammer Active        Pending ☒ None       

a. Source and identifying no. National Institutes of Health P.I. Steven Soper

Title DNA Sequencing Using Heavy-Atom Modified Near-IR Dyes

b. Your role on project Co-Principal Investigator % Effort 10%

c. Dates and costs of entire project April. 1, 1994 - Mar. 30, 1999; \$ 485,075

d. Dates and costs of current year       

e. Specific aims of project The Aims of this project are the same as for LEQSF 066A.

f. Describe scientific and budgetary overlap There is significant scientific and budgetary overlap with the

LEQSF 066A grant and the DOE application. No significant overlap with the current application.

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

No adjustments necessary.

**OTHER SUPPORT**

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Name Robert P. Hammer Active            Pending X None           

a. Source and identifying no. USAMRDC (Log number 92148004) P.I. David H. Swenson

Title Hyperstabilizing Antisense Oligonucleotides

b. Your role on project Co-Principal Investigator % Effort 10%

c. Dates and costs of entire project Sept. 1, 1993 - Aug. 30, 1996; \$787,304

d. Dates and costs of current year Sept. 1, 1993 - Aug. 30, 1994; \$267,704

e. Specific aims of project The Aims of this project are:

1. Synthesis of tethers and ligands, based on CC-1065, expected to hyperstabilize DNA duplexes, DNA-RNA hybrids, and synthesis of the duplexes or hybrids; 2. Evaluation of take-up of phosphate-modified antisense oligonucleotides by bacteria related to *Bacillus anthracis*. direct comparison of properties to analogous DNA or RNA oligomers; 3. Evaluation of the hyperstabilizing effects of tethered and non-tethered ligands with DNA-DNA and DNA-RNA duplexes; 4. Characterize the hyperstabilizing effect at molecular and atomic level by NMR and molecular modeling and computational studies; 5. Characterize the the ability of select ligand-oligonucleotide conjugates to inhibit translation of *Bacillus subtilis* in vitro and in vivo; 6. Characterize the uptake and distribution of modified antisense oligonucleotides in mammalian inhalation and intravenous experiments.

f. Describe scientific and budgetary overlap No scientific or budgetary overlap

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

No adjustments will be necessary



### OTHER SUPPORT

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Name Matthew B. Lubin Active \_\_\_\_\_ Pending X None \_\_\_\_\_

a. Source and identifying no. US Army Med. Rsch. and Devel. Command P.I. Barany, F.

Title Multiplex Detection of Point Mutations, Amplifications and Deletions in Breast Cancer

b. Your role on project C0- Investigator % Effort 10%

c. Dates and costs of entire project 09/01/94 to 08/31/98; \$469,978 (Co-I's portion; \$164,492)

d. Dates and costs of current year \_\_\_\_\_

e. Specific aims of project To develop new approaches for the simultaneous detection of genetic alterations in breast cancer by developing: i) a multiplex polymerase chain reaction/ligase detection reaction (PCR/LDR) system for detecting point mutations in tumors; ii) a multiplex ligase detection reaction/polymerase chain reaction (LDR/PCR) system to detect gene amplifications and deletions in tumors; and iii) a polymerase chain reaction/restriction endonuclease/ligase detection reaction (PCR/RE/LDR) system to detect cancer cells present at a level of only 1 in 10<sup>6</sup> cells in clinical specimens.

f. Describe scientific and budgetary overlap There will be some scientific and budgetary overlap. This DOD proposal is a pilot version of the more detailed experiments described in Project 2.

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

Some adjustments will be necessary.

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Name Matthew B. Lubin, MD Active \_\_\_\_\_ Pending X None \_\_\_\_\_

a. Source and identifying no. National Cancer Institute P.I. Bovberg, D.

Title Psychoimmune mechanisms in women's familial cancer risk

b. Your role on project PI of subcontract—Strang Cancer Prev. Ctr. % Effort 5%

c. Dates and costs of entire project 95/07/01 to 97/06/30 ; \$ 765,448 (M.L. portion \$17,602)

d. Dates and costs of current year \_\_\_\_\_

e. Specific aims of project The aim of this project is to identify and characterize NK cell

activity abnormalities in subsets of women at risk for breast cancer because of  
their family histories of cancer.

f. Describe scientific and budgetary overlap No Scientific or budgetary overlap

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

No adjustments will be necessary.

**OTHER SUPPORT**

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Name Vincent L. Wilson Active X Pending \_\_\_\_\_ None \_\_\_\_\_

a. Source and identifying no. Univ. Colorado Lung Cancer SPORE P.I. Vincent L. Wilson

Title Detection and identification of oncogenic point mutations in human lung tumors

b. Your role on project Principal Investigator % Effort 25%

c. Dates and costs of entire project 93/10/01 to 94/09/30 ; \$ 25,000

d. Dates and costs of current year same (pilot project)

e. Specific aims of project The Aims of this Project are:

1) To determine the frequency of point mutations in the oncogenic loci, Ha-ras codon 12 and p53 codon 248, in lung tumor and normal adjacent tissue, and in sputum specimens.

2) Develop PCR/LCR assays for other relevant loci, e.g. Ki-ras and N-ras codons 12, 13, and 61, and hotspot codons in p53. 3) Compare these mutation frequencies with other markers of tumor induction, tumor type, clinical history, and the clinical evaluation of the patients.

f. Describe scientific and budgetary overlap This project overlaps the present proposal in the development of MspI and non-thermostable restriction enzyme based PCR/LCR assays in oncogenic loci in the search for markers of tumor induction in lung cancer. However, this is a pilot project only and support ends September, '94.

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

No Adjustments will be necessary

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Name Vincent L. Wilson Active X Pending \_\_\_\_\_ None \_\_\_\_\_

a. Source and identifying no. University of Colorado Cancer Center P.I. Marie E. Wood, M.D.

Title Multidisciplinary evaluation of cancer families

b. Your role on project Co- Investigator % Effort 25%

c. Dates and costs of entire project 93/10/01 to 94/09/30 ; \$ 49,857

d. Dates and costs of current year same (pilot project)

e. Specific aims of project The Aims of this Project are: 1) To determine if accurate detection of Li-Fraumeni families is possible by identifying families at risk through pediatric cancer cases; 2) To develop screening protocols for early diagnosis of malignancies commonly associated with the Li-Fraumeni syndrome; and 3) To identify, through molecular techniques, individuals from Li-Fraumeni families who appear to carry germline mutations in p53.

f. Describe scientific and budgetary overlap There is no budgetary overlap. However, this project is substantially supportive of the present proposed research directives. The identification of cancer families and heritable germline mutations will only advance the ability of this laboratory to continue to guide the study of oncogenic mutations, as well as provide a population of people carrying predisposing mutations.

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

No Adjustments will be necessary

## TABLE OF OTHER SUPPORT FOR INVESTIGATORS

Investigator	Source/ID	% effort	Current year	Comments
A. AGGARWAL	NIH 5 R01-GM44006-04	50%	\$99,284	Expires 12/94
	NIH 1 R01 GM49327-01	15%	\$124,699	
	Hischl Faculty Award	-	\$20,000	
	ACS NP-75182	10%	\$69,484	
	TOTAL (Current)	75%		
F. BARANY	NIH 5 R01 GM41337-05	50%	\$122,960	Expires 07/94 P.I. Salary
	Hirschl/Weill-Caulier Award	-	\$20,000	
	Applied Biosystems	10%	\$126,500	
	Roche Molecular Systems	5%	\$56,300	Pending review Pending review Needs adjustment
	NIH 5 R01 GM41337-06	40%	\$200,000	
	US Army Research	10%	\$75,000	
	TOTAL (Current)	65%		
G. BARANY	NIH GM43552-04	26%	\$91,574	
	NIH GM42722-05	18%	\$144,598	
	NIH GM51628-01	18%	\$167,246	
	Selectide Corp.	5%	\$70,000	
	TOTAL (Current)	67%		
D. BERGSTROM	NIH AI26029-07	10%	\$95,870	Expires 06/94
	Walther Cancer Inst.	40%	\$100,000	Expires 06/94
	NCI 5-T32-CA09634-04	-	\$15,000	Student stipend
	NIH R01 AI36601-01	10%	\$120,000	Pending review
	NIH STRR 94-1	10%	\$12,384	Pending review
	NIH SBIR 93-25	10%	\$14,098	Pending review
	TOTAL (Current)	50%		
N. HACKETT	NIH R29HG00349-05	50%	\$40,000	Expires 07/94
	Cornell Scholarship	-	\$30,000	Expires 06/94
	NIH RFA AI93-023	35%	\$110,000	Pending review
	Heiser Foundation	40%	\$20,000	Pending review
	NSF-Biochemistry	40%	\$100,000	Pending review
	TOTAL (Current)	50%		Need adjustment
R. HAMMER	ACS PRF #26716-G4	10%	\$10,000	Pending review Pending review
	Louisiana Education Fund	15%	\$35,000	
	Louisiana Education Fund	10%	\$64,559	
	NIH Human Genome Prog.	10%	\$90,000	
	Army MRDC(92148004)	10%	\$267,704	
	TOTAL (Current)	35%		
M. LUBIN	US Army MRDC	10%	\$40,000	Pending review
	NCI	5%	\$17,602	Pending review
	TOTAL (Current)	0%		
V. WILSON	U. Colorado SPORE	25%	\$25,000	
	U. CO. Cancer Center	25%	\$49,857	
	TOTAL (Current)	50%		

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME Aneel K. AGGARWAL	POSITION TITLE Assistant Professor
---------------------------	---------------------------------------

EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Kings College, University of London, England	B.Sc.	1981	Biology & Physics
Kings College, University of London, England	Ph.D.	1984	Biophysics
Harvard University, Cambridge, MA	Postdoc	1984-89	Biochem & Mol. Biol.

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

1984-89 Postdoctoral Fellow, Dept. of Biochemistry and Molecular Biology,  
Harvard University

1989-present Assistant Professor, Department of Biochemistry and Molecular Biophysics,  
Columbia University

*Honors and Awards:*

1981 B.Sc. with First Class Honors

1981 Flowers Memorial Prize for Physics, Kings College

1981 Layton Science Research Prize, Kings College

1981-84 Predoctoral Scholarship, Science and Engineering Research Council  
of Great Britain

1985-87 NATO Research Fellowship

1990-95 Irma T. Hirschl Career Scientist Award

1990-92 Basil O'Connor Starter Scholar Research Award

*Representative Publications:*

A.K. Aggarwal, D.W. Rodgers, M. Drott, M. Ptashne, and S.C. Harrison (1988). Recognition of DNA operator by the repressor of phage 434: A view at high resolution. *Science* 242, 899-907.

C.O. Pabo, A.K. Aggarwal, S. Jordan, L. Beamer, U. Obeysekare, and S.C. Harrison (1990). Conserved residues make similar contacts in two repressor-operator complexes. *Science* 247, 1210-1213.

S.C. Harrison and A.K. Aggarwal (1990). DNA recognition by proteins with the helix-turn-helix motif. *Ann. Rev. Biochem.* 59, 933-969.

A.K. Aggarwal (1990). Crystallization of DNA binding proteins with oligodeoxynucleotides.  
*Methods* 1, 83-90

W.E. Jack, L. Greenough, L.F. Dorner, S-Y Xu, T. Strzelecka, A.K. Aggarwal, and I. Schildkraut  
(1991) Overexpression, purification and crystallization of *Bam*HI endonuclease.  
*Nucl. Acids Res.* 19, 1825-1829

L. Peng, X. He, M.R. Gerrero, M. Mok, A.K. Aggarwal, and M.G. Rosenfeld (1993) Spacing and  
orientation of bipartite DNA-binding motifs as potential functional determinants for POU  
domain factors. *Genes and Dev.* 7, 2483-2496

M. Newman, T. Strzelecka, L.F. Dorner, I. Schildkraut, and A.K. Aggarwal (1993) The structure of  
restriction endonuclease *Bam*HI and its relationship to *Eco*RI. *Nature* - Manuscript submitted

T. Strzelecka, M. Newman, L.F. Dorner, I. Schildkraut, and A.K. Aggarwal (1993) Crystallization  
and preliminary X-ray analysis of restriction endonuclease *Bam*HI-DNA complex.  
*J. Mol. Biol.* - Manuscript submitted.

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME		POSITION TITLE	
Dennis J. AHNEN		Associate Professor	
EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Florida State University, Tallahassee, FL	B.S.	1969	Biology
Wayne State University, Detroit, MI	M.D.	1973	Medicine

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

Medical Intern, Hutzel Hospital, Detroit, MI	1973-74
Medical Resident, Hutzel Hospital, Detroit, MI	1974-77
Chief Medical Resident, Hutzel Hospital, Detroit, MI	1976-77
Fellow in Gastroenterology, UCHSC, Denver, CO	1977-80
Membrane Pathobiology Fellow, Stanford Med. School, Stanford, CA	1980-81
Visiting Scholar, Stanford University, Stanford, CA	1981-82
Assistant Professor, UCHSC, Denver, CO	1982-87
Research Associate, Denver VA Medical Center, Denver, CO	1984-87
Associate Professor, UCHSC, Denver, CO	1987-Present
Clinical Investigator, Denver VA Medical Center, Denver, CO	1990-95
Research Leader for Cancer Prevention and Control, University of Colorado Cancer Center, Denver, CO	

*Representative Publications : (from a total of 58 published papers):*

- Ahnen, D.J., Nakane, P.K., & Brown, W.R.: Ultrastructural localization of carcinoembryonic antigen in normal intestine and colon cancer. Abnormal distribution of CEA on the surfaces of colon cancer cells. *Cancer* 49:2077, 1982.
- Ahnen, D.J. & Brown, W.R.: Campylobacter enteritis in immune-deficient patients. *Ann. Int. med.* 96:187, 1982.
- Ahnen, D.J., Santiago, N.A., Cezard, J.P., & Gray, G.M.: Intestinal aminooligopeptidase: In vivo synthesis on intracellular membranes of rat jejunum. *J. Biol. Chem.* 257:121-129, 1982.
- Ahnen, D.J., Mircheff, A.K., Santiago, N.A., Yoshioka, C., & Gray, G.M.: Intestinal surface aminooligopeptidase: Distinct molecular forms during assembly on intracellular membranes in vivo. *J. Biol. Chem.* 258:5960, 1983.
- Mircheff, A.K., Ahnen, D.J., Islam, A., Santiago, N.A., & Gray, G.M.: Complex subcellular distribution of enzymatic markers in intestinal epithelial cells. *J. Membr. Biol.* 83:95-107, 1985.
- Boland, C.R. & Ahnen, D.J.: Peanut lectin labeling of colonic mucin occurs in response to carcinogen prior to the appearance of neoplastic lesions. *Gastroenterology* 89:127-137, 1985.
- Ahnen, D.J., Singleton, J.R., Hoops, T.C., & Kloppel, T.M.: Postranslational processing of secretory component in the rat jejunum by a brush border metalloprotease. *J. Clin. Invest.* 77:1841-1848, 1986.



- Shioda, Y., Brown, W.R., & Ahnen, D.J.: Serial observations of colonic carcinogenesis in the rat. Premalignant mucosa binds *Ulex europaeus* agglutinin. *Gastroenterology* 92:1-12, 1987.
- Meltzer, S.J., Ahnen, D.J., Battifora, H., Yakota, J., & Cline, M.J.: Protooncogene abnormalities in colon cancers and adenomatous polyps. *Gastroenterology* 92:1174-1180, 1987.
- Ahnen, D.J., Kinoshita, Nakane, P.K., & Brown, W.R.: Differential expression of carcinoembryonic antigen (CEA) and secretory component (SC) during colonic epithelial cell differentiation and in colon carcinoma. *Gastroenterology* 93:1330-1338, 1987.
- Ahnen, D.J., Greene, C., Warren, G., & Brown, W.R.: Mucin histochemistry of dysplasia in ulcerative colitis (UC): Search for specific markers. *Gastroenterology* 93:1346-1355, 1987.
- Sugiyama, T., Brown, W.R., & Ahnen, D.J.: Expression of cell surface antigens on rat colonic cancer cells. *Gastroenterology* 94:331-342, 1988.
- Ahnen, D.J., Reed, T.A., Bozdech, J.M.: Isolation and characterization of populations of mature and immature colonocytes. *Am. J. Physiol.* 254:G610-G621, 1988.
- Thacte, L.G., Ahnen, D.J., & Malkinson, A.M.: Proliferating cell nuclear antigen (PCNA/cyclin) immunocytochemistry as a labeling index in mouse lung tissue. *Cell and Tissue Res.* 256:167-173, 1989.
- Molitoris, B.A., Hoilien, C.A., Dahl, R., Ahnen, D.J., Wilson, P.D., & Kin, J.: Characterization of ischemia-induced loss of epithelial polarity. *J. Membr. Biol.* 106:233-242, 1988.
- Everson, G.E., Ahnen, D.J., Harper, P.C., & Krawitt, E.L.: Benign recurrent intrahepatic cholestasis: Treatment with S-adenosylmethionine. *Gastroenterology* 96:1354-1357, 1989.
- Yamada, K., Yoshitake, K., Sato, M., & Ahnen, D.J.: Proliferating cell nuclear antigen expression in normal preneoplastic and neoplastic colonic epithelium of the rat. *Gastroenterology* 103:160-167, 1992.
- Sato, M. & Ahnen, D.J.: Regional variability in colonic growth regulation in the rat. *Anat. Record* 233:409-414, 1992.
- Spechler, S.J., Ahnen, D.J., and the Department of Veterans Affairs Gastroesophageal Reflux Disease Study Group: Comparison of medical and surgical therapy for complicated gastroesophageal reflux in veterans. *N.Engl. J. Med.* 326:786-792, 1992.
- Hauft, S.M., Schmidt, G.H., Pease, S., Roth, K.A., Hanbrough, J.R., Cohn, S.M., Ahnen, D.J., Wright, N.A., Goodlad, R.A., & Gordon, J.I.: Expression of SV40 T antigen in the small intestinal epithelium of transgenic mice results in re-entry of differentiating villus-associated enterocytes into the cell cycle without an apparent effect on the differentiation program and without causing neoplastic transformation. *J. Cell Biol.* 177:825-839, 1992.
- Wright, N.A., Poulosom, R., Stamp, G., Van Norden, S., Elia, G., Ahnen, D.J., Jeffrey, R., Longcroft, J., Pike, C., Rio, M.-C., & Chambon, P.: Tefoil peptide gene expression in gastrointestinal epithelial cells in inflammatory bowel disease. *Gastroenterology* 104:12-20, 1993.
- Ahnen, D.J., Poulosom, R., Stamp, G.W.H., Elia, G., Pike, C., Jeffrey, R., Longcroft, J., Rio, M.-C., Chambon, P., & Wright, N.A.: The ulceration-associated cell lineage (UACL) reiterates the Brunner's gland differentiation program but acquires the proliferative organization of the gastric gland. (Submitted)

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME Francis BARANY		POSITION TITLE Associate Professor / Principal Investigator	
EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
University of Illinois at Chicago Circle, Chicago	B.A.	1976	Chemistry
The Rockefeller University, New York	Ph.D.	1981	Microbiology
The Rockefeller University, New York	Postdoc.	81-82	Microbiology
The Johns Hopkins Univ. Sch. of Medicine, Baltimore	Postdoc	82-85	Molecular Biology

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

**Professional Experience:**

1981-1982 The Rockefeller University, Postdoctoral Fellow; A. Tomasz  
 1982-1985 The Johns Hopkins University School of Medicine, Postdoctoral Fellow; H.O. Smith  
 1985-1990 Cornell University Medical College, Assistant Professor, Dept. of Microbiology  
 1990-present Cornell University Medical College, Associate Professor, Dept. of Microbiology  
 1986-present The Rockefeller University, Adjunct Assistant, Associate Professor

**Honors and Awards:**

Westinghouse Science Talent Search, National Finalist, 1974  
 B.B. Freund Award for Excellence in Chemistry, 1975  
 The American Institute of Chemists Award, 1976  
 National Science Foundation Predoctoral Fellowship, 1976-1979  
 Rockefeller University Graduate Fellowship, 1980-1981  
 Andrew W. Mellon Postdoctoral Fellow, 1981-1982  
 Helen Hay Whitney Fellow, 1982-1985  
 Cornell Scholar in Biomedical Sciences, 1985-1988  
 Hirschl/Monique Weill-Caulier Career Scientist Award, 1992-1997

**Representative Publications:** (excerpted from a total of over 35 refereed papers)

- Barany, F. (1987) A genetic system for isolation and characterization of *TaqI* restriction endonuclease mutants. *Gene* 56: 13-27.
- Barany, F. (1988) The *TaqI* star reaction: strand preferences reveal hydrogen bond donor and acceptor sites in canonical sequence recognition. *Gene* 65: 149-165.
- Barany, F. (1988) Overproduction, purification, and crystallization of *TaqI* restriction endonuclease. *Gene* 65: 167-177.
- Glushka, J., Barany, F., & Cowburn, D. (1989) Observation of arginyl-deoxyoligonucleotide interactions in *TaqI* endonuclease by detection of specific  $^1\text{H}$  NMR signals from 140kD [Nn1, Nn2,  $^{15}\text{N}$  Arg] *TaqI*/oligomer complexes. *Biochem. & Biophys. Res. Commun.* 164:88-93.
- Barany, F. (1991) Genetic disease detection and DNA amplification using cloned thermostable ligase. *Proc. Natl. Acad. Sci. USA*, 88:189-193.

- Barany, F. (1991) The ligase chain reaction (LCR) in a PCR world. *PCR Methods and Applications* 1: 5-16.
- Zebala, J., & Barany, F. (1991) Mapping catalytically important regions of an enzyme using two-codon insertion mutagenesis: a case study correlating  $\beta$ -lactamase mutants with the three dimensional structure. *Gene* 100:51-57.
- Barany, F. and Gelfand, D. (1991) Cloning, overexpression, and nucleotide sequence of a thermostable DNA ligase-encoding gene. *Gene* 109:1-11.
- Barany, F., Danzitz, M., Zebala, J. and Mayer, A. (1992) Cloning and sequencing of genes encoding the *Tth*HB8I restriction and modification enzymes; comparison with the isoschizomeric *Taq*I enzymes. *Gene* 112:3-12.
- Barany, F. and Zebala, J. (1992) Correlation between insertion mutant activities and amino acid identities of the *Taq*I and *Tth*HB8I restriction endonucleases. *Gene* 112:13-20.
- Barany, F., Slatko, B., Danzitz, M., Cowburn, D., Schildkraut, I. and Wilson, G. (1992) The corrected nucleotide sequence of the *Taq*I restriction and modification enzymes reveals a thirteen-codon overlap. *Gene* 112:91-95.
- Zebala, J., Choi, J. and Barany, F. (1992) Characterization of steady state, single-turnover and binding kinetics of the *Taq*I restriction endonuclease. *J. Biol. Chem.* 267: 8097-8105.
- Zebala, J., Choi, J., Trainor, G. and Barany, F. (1992) DNA recognition of base-analogue and chemically modified substrates by the *Taq*I restriction endonuclease. *J. Biol. Chem.* 267:8106-8116.
- Wiedman, M., Czajka, J., Barany, F. and Batt, C. (1992) Discrimination of *Listeria monocytogenes* from other *Listeria* species by ligase chain reaction. *Appl. Environ. Microbiol.* 58:3443-3447.
- Zebala, J. and Barany, F. Detection of Leber's Hereditary Optic Neuropathy by nonradioactive-LCR. In *PCR Protocols: A guide to methods and applications* 2nd edition, M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White eds., Academic Press, San Diego, In press, August 4, 1992.
- Prchal, J.T., Guan, Y.L., Prchal, J.F. and Barany, F. (1993) Transcriptional analysis of the active X-chromosome in normal and clonal hematopoiesis. *Blood* 81: 269-271.
- Feero, W.T., Wang, J., Barany, F., Zhou, J., Todorovic, S.M., Conwit, R., Galloway, G., Hausmanowa-Petrusewicz, I., Fidzianska, A., Arahata, K., Wessel, H.B., Wadelius, C., Marks, H.G., Hartlage, P., Hayakawa, H., and Hoffman, E.P. (1993) Hyperkalemic Periodic Paralysis: Rapid molecular diagnosis and relationship of genotype to phenotype in 12 families. *Neurology* 43:668-673.
- Wang, J., Zhou, J., Todorovic, S.M., Feero, W.G., Barany, F., Conwit, R., Hausmanowa-Petrusewicz, I., Fidzianska, A., Arahata, K., Wessel, H.B., Sillen, A., Hayakawa, H., and Hoffman, E.P. (1993) Molecular genetic and genetic correlations in sodium channelopathies: Lack of founder effect and evidence of a second gene. *Am. J. Hum. Genet.* 52: 1074-1084.
- Zebala, J., and Barany, F. (1993) Implications for the ligase chain reaction in gastroenterology. *J. Clin. Gastroenterol.* 17:171-175.
- Wiedman, M., Barany, F. and Batt, C. (1993) Detection of *Listeria monocytogenes* with a non-isotopic polymerase chain reaction-coupled ligase chain reaction assay. *Appl. Environ. Microbiol.* 59:2743-2745.
- Wiedman, M., Wilson, W., Czajka, J., Luo, J., Barany, F., Batt, C. (1994) Ligase chain reaction (LCR)-Overview and applications. (Invited Review) *PCR Methods and Applications*. In press.

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME George BARANY		POSITION TITLE Professor of Chemistry / Principal Investigator Project 5	
EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
The Rockefeller University, New York. (admitted to graduate program in 1971 directly from high school)	Ph.D	1977	Biochemistry, Mathematics, Organic Chemistry
The Rockefeller University, New York	Postdoc	1977-80	Peptide Synthesis

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

University of Minnesota, Assistant Professor of Chemistry, 1980-1986.

University of Minnesota, Associate Professor of Chemistry, 1986-1991.

University of Minnesota, Professor of Chemistry, 1991-present.

*Research Interests and Experience:*

solid-phase peptide synthesis, orthogonal protection, mechanism of protein folding, organosulfur chemistry, polymer functionalization chemistry, various spectroscopic techniques

*Relevant Professional Activities:*

Consultant on new methods of peptide synthesis, Millipore Corporation, Bedford, Massachusetts (originally MilliGen/Biosearch in Novato, California), 1985-present.

Steering committee, Univ. of Minnesota Microchemical Facility, 1985-present.

*Ad hoc* member, Bio-organic and natural products chemistry study section,

National Institutes of Health, February 1988 and June 1991.

Consultant on multiple syntheses and peptide drug discovery, Arris Pharmaceutical Corporation, South San Francisco, California, 1992-present.

Editorial Board, *Int. J. Peptide Protein Res.*, 1992-present.

Steering committee, Univ. of Minnesota Biomedical Engineering Center Mass Spectrometry Research Facility, 1992-present.

NSF, Chemistry Division, Postdoctoral fellowship review panel, February 1993.

Medical Research Council (Canada), Site visit team, March 1993.

Member, Multidisciplinary special emphasis study section to review SBIR grant applications, National Institutes of Health, March 1993.

Council, American Peptide Society, 1993-present (elected for 6 year term).

*Honors:*

The Rockefeller University Graduate Fellowships, 1972-1977.

USPHS Postdoctoral Fellowship, 1978-1980.

Searle Scholars Program, 1982-1985.

USPHS Research Career Development Award, 1982-1987.

"America's 100 Brightest Scientists under 40," *Science Digest* survey, December, 1984.

"Frontiers in Science" lecturer, Carlsberg Research Laboratories, Copenhagen, Denmark, Oct. 1991.

Vincent du Vigneaud Award, for contributions to the chemistry and/or biology of peptides, 1994.

*Representative Publications:* (excerpted from total of over 100 refereed papers and reviews)

- G. Barany and R.B. Merrifield. Solid-Phase Peptide Synthesis. In "The Peptides" (E. Gross and J. Meienhofer, eds.), Volume 2, Academic Press, New York, 1979, pp. 1-284.
- N. Kneib-Cordonier, F. Albericio, and G. Barany. Orthogonal solid-phase synthesis of human gastrin-I under mild conditions. *Int. J. Peptide Protein Res.* **35**, 527-538 (1990).
- R.P. Hammer, F. Albericio, L. Gera, and G. Barany. Practical approach to solid-phase synthesis of C-terminal peptide amides under mild conditions based on a photolysable anchoring linkage. *Int. J. Peptide Protein Res.* **36**, 31-45 (1990).
- F. Albericio, R.P. Hammer, C. García-Echeverría, M.A. Molins, J.L. Chang, M.C. Munson, M. Pons, E. Giralt, and G. Barany. Cyclization of disulfide-containing peptides in solid-phase synthesis. *Int. J. Peptide Protein Res.* **37**, 402-413 (1991).
- F. Albericio and G. Barany. Hypersensitive Acid-Labile (HAL) Tris(alkoxy)benzyl Ester Anchoring for Solid-Phase Synthesis of Protected Peptide Segments. *Tetrahedron Lett.* **32**, 1015-1018 (1991).
- G.B. Fields, Z. Tian, and G. Barany. Principles and Practice of Solid-Phase Peptide Synthesis. In "Synthetic Peptides: A User's Guide" (G.A. Grant, ed.), Chapter 3, W.H. Freeman & Co., New York, invited review article, 1992, pp. 77-183.
- G. Barany, N.A. Solé, R.J. Van Abel, F. Albericio, and M. E. Selsted. Recent Advances in Solid-Phase Peptide Synthesis. In "Innovation and Perspectives in Solid Phase Synthesis and Related Technologies: Peptides, Polypeptides and Oligonucleotides 1992" (R. Epton, ed.), Intercept, Andover, England, 1992, pp. 135-152.
- N.A. Solé and G. Barany. Optimization of Solid-Phase Synthesis of [Ala<sup>8</sup>]-dynorphin A. *J. Org. Chem.* **57**, 5399-5403 (1992).
- S.A. Kates, N.A. Solé, C.R. Johnson, D. Hudson, G. Barany, and F. Albericio. A Novel Convenient Three-Dimensional Orthogonal Strategy for Solid-Phase Synthesis of Cyclic Peptides. *Tetrahedron Lett.* **34**, 1549-1552 (1993).
- E.A. Ottinger, L.L. Shekels, D.A. Bernlohr, and G. Barany. Synthesis of Phosphotyrosine-Containing Peptides and their Use as Substrates for Protein Tyrosine Phosphatases. *Biochemistry* **32**, 4354-4361 (1993).
- M.C. Munson and G. Barany. Synthesis of  $\alpha$ -Conotoxin SI, a Bicyclic Tridecapeptide Amide with Two Disulfide Bridges: Illustration of Novel Protection Schemes and Oxidation Strategies. *J. Am. Chem. Soc.* **115**, 10203-10216 (1993).
- D. Andreu, F. Albericio, N.A. Solé, M.C. Munson, M. Ferrer, and G. Barany. Formation of Disulfide Bonds in Synthetic Peptides and Proteins. In "Peptide Synthesis and Purification Protocols" (M.W. Pennington and B.M. Dunn, Eds.), Humana Press, Clifton, N.J., in press (1994).
- J. Vágner, V. Krchňák, N.F. Sepetov, P. Štrop, K.S. Lam, G. Barany, and M. Lebl. Novel Methodology for Differentiation of "Surface" and "Interior" Areas of Polyoxyethylene-Polystyrene (POE-PS) Supports: Application to "Library" Screening Procedures. In "Innovation and Perspectives in Solid Phase Synthesis and Related Technologies" (R. Epton, ed.), SPCC (UK) Ltd., Birmingham, in press (1994).
- S. Zalipsky, J.L. Chang, F. Albericio, and G. Barany. Preparation and Applications of Polyethylene Glycol-Polystyrene Graft Resin Supports for Solid-Phase Peptide Synthesis. *Reactive Polymers*, in press (1994).

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME Carl A. BATT	POSITION TITLE Associate Professor
----------------------	---------------------------------------

EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Kansas State University	B.S.	1975	Microbiology
Rutgers University	M. S.	1979	Food Science
Rutgers University	Ph.D.	1981	Food Science
Massachusetts Institute of Technology	Postdoc	81-85	Applied Biology

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

**Professional Experience:**

- 1982-1984 Postdoctoral Research Associate, Department of Nutrition and Food Science  
Massachusetts Institute of Technology
- 1984-1985 Research Scientist, Department of Applied Biological Sciences  
Massachusetts Institute of Technology
- 1985-1991 Assistant Professor, Department of Food Science, Cornell University
- 1991-present Associate Professor, Department of Food Science, Cornell University

**Honors and Awards:**

- 1988-present Yoplait Institute Research Award
- 1990-present Samuel Cate Prescott Award (IFT)

**Representative Publications (Excerpted from over 100 publications):**

- Batt, C.A., Jamieson, A.J. and Vandeyar, M.A. (1990) Identification of essential histidine residues in the active site of the *Escherichia coli* xylose (glucose) isomerase. *Proc. Natl. Acad. Sci. USA* 87, 618-622.
- Batt, C.A., Rabson, L.D., Wong, D.W.S. and Kinsella, J.E. (1990) Expression of recombinant bovine  $\beta$ -lactoglobulin in *Escherichia coli*. *Agr. Biol. Chem.* 54, 949-955.
- Silva, M, Wong, D.W.S. and Batt, C.A. (1990) Cloning and sequencing of the genomic bovine  $\beta$ -lactoglobulin gene. *Nucl. Acid. Res.* 18, 3051.
- Kim, S.G. and Batt, C.A. (1991) Identification of a nucleotide sequence conserved in *Lactococcus lactis* bacteriophages. *Gene* 98, 95-100.
- Kim, S.G. and Batt, C.A. (1991) Antisense mRNA mediated bacteriophage resistance in *Lactococcus lactis*. *Appl. Environ. Micro.* 57, 1109-1113.
- Chung, D.K., Kim, J.H. and Batt, C.A. (1991) Molecular cloning and nucleotide sequence of the major capsid protein of *Lactococcus lactis* ssp. *cremoris* bacteriophage F4-1. *Gene* (in press).
- Gavalchin, J., Tortorello, M.L., Malek, R., Landers, M. and Batt, C.A. (1991) Isolation of monoclonal antibodies that react preferentially with *Listeria monocytogenes*. *Food Microbiol.* 8, 325-330.

- Bor, Y-C., Moraes, C., Lee, S-P., Crosby, W., Sinskey, A.J. and Batt, C.A. (1992) Cloning and nucleotide sequence of the *Lactobacillus brevis* xylose isomerase. *Gene* 114, 127-131.
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- Wiedmann, M., Czajka, J., Barany, F. and Batt, C.A. (1992) Discrimination of *Listeria monocytogenes* from other *Listeria* species by ligase chain reaction. *Appl. Environ. Microbiol.* 58, 3443-3447.
- Batt, C.A., Cho, Y. and Jamieson, A.C. (1993) A simple and rapid method for the selection of oligodeoxynucleotide-directed mutants. *Meth. Enzymol.* 217, 280-286.
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- Kim, S. G. and Batt, C. A. (1993) Cloning and sequencing the *Lactococcus lactis* subsp. *lactis* *groESL* operon. *Gene* 127, 121-126.
- Winn-Deen, E., Batt, C.A. and Wiedmann, M. (1993) Non-radioactive detection of *Mycobacterium tuberculosis* LCR products in a microtitreplate format. *Mol. Cell Probes* 7, 179-186.
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- Wiedmann, M., Barany, F. and Batt, C.A. (1993) Detection of *Listeria monocytogenes* using a nonisotopic polymerase chain reaction (PCR)-couple ligase chain reaction (LCR) assay. *Appl. Environ. Microbiol.* 59, 2743-2745.
- Lee, S.P., Kim, D.S., Watkins, S. and C.A. Batt. (1993) Reducing whey syneresis in yogurt by the addition of a thermolabile variant of  $\beta$ -lactoglobulin. *Biosci. Biotechnol. Biochem.* (submitted)
- Cho, Y., Batt, C.A. and Sawyer, L. (1993) Probing the retinol binding site of bovine  $\beta$ -lactoglobulin. *Biochemistry* (submitted).
- Wiedmann, M., Luo, J., Barany, F. and Batt, C.A. (1993) Detection of *Listeria monocytogenes* by PCR coupled ligase chain reaction (LCR). *PCR Protocols* (accepted).
- Wilson, W.J., Wiedmann, M., Dillard, H.R. and Batt, C.A. (1993) Identification of *Erwinia stewartii* by a ligase chain reaction. *Appl. Environ. Microbiol.* (submitted)
- Wiedmann, M., Wilson, W., Czajka, J., Barany, F. and Batt, C.A. (1993) Ligase-mediated detection techniques. Second Symposium on Usage of PCR and Alternative DNA Amplification Methods in Genetic and Infectious Diseases. (accepted)
- Batt, C.A., Wagner, P., Wiedmann, M., Luo, J., Gilbert, R. (1993) Detection of bovine leukocyte adhesion deficiency by nonisotopic ligase chain reaction. *Anim. Genet.* (submitted)
- Cha, J., Cho, Y., Whitaker, R.D., Carrell, H.L., Glusker, J.P., Karplus, P.A., and Batt, C.A. (1993) Perturbing the metal site in D-xylose isomerase: The effect of mutation of His-220 on enzyme stability. *J. Biol. Chem.* (submitted).

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME Donald E. BERGSTROM	POSITION TITLE Professor of Medicinal Chemistry
-----------------------------	--

EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
University of Washington, Seattle, WA	B.S.	1965	Chemistry
University of California, Berkeley, CA	Ph.D.	1970	Organic Chemistry
University of Illinois, Urbana, IL	Postdoc.	70-72	Chemistry

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

1972-1974 Assistant Professor, Rockefeller University, New York, New York  
 1974-1980 Assistant Professor, University of California, Davis  
 1980-1985 Associate Professor, University of North Dakota  
 1986-1989 Professor, University of North Dakota  
 1989-present Professor of Medicinal Chemistry, Purdue University  
 1992-present Deputy Director, Purdue Cancer Center

*Honors and Awards:*

1985 Sigma Xi Scientific Research Society Faculty Award for Outstanding Research

*Representative Publications:*

- Bergstrom, D. E., Beal, P., Husain, A., Lind, R., and Jenson J., "Palladium-Mediated Coupling Between Organic Disulfides and Nucleic Acid Constituents," *J. Am. Chem. Soc.* **1989**, *111*, 374-375.
- Bergstrom, D. and Schmaltz, T., "Synthesis of (Dicarbonyl)(h<sup>5</sup>-cyclopentadienyl)iron-Derived Nucleoside Phosphonate Esters," *Nucleosides and Nucleotides* **1989**, *8*, 1057-1059.
- Bergstrom, D., Beal, P., and Lind, R., "Synthesis of (Dicarbonyl)(h<sup>5</sup>-cyclopentadienyl)-Manganese Complex Stabilized Nucleoside Phosphite Esters," *Nucleosides and Nucleotides* **1989**, *8*, 1061-1063.
- Bergstrom, D. E., Lind, R.E., Mott, A. W., and Swartling, D. J., "Preparation of 3'-Keto-5'-O-tritylthymidine," *Nucleosides and Nucleotides* **1989**, *8*, 1529-1535.
- Bergstrom, D. E., Abrahamson, J. K., and Chan, M. Y.-M., "Nucleoside and Nucleotide Transport Through a Model Liquid Membrane. Periodic-Catastrophic Transport of a Novel Amantadine Phosphoramidate Conjugate of 5'-AMP," *Biochemica Biophysica Acta* **1991**, *1061*, 95-105.
- Bergstrom, D. E., Abrahamson, J. K., and Chan, M. Y.-M., "Periodic-Catastrophic Transport of an Adenosine 5'-Monophosphate Amantadine Conjugate Through a Model Liquid Membrane," *Nucleosides and Nucleotides* **1991**, *10*, 685-688.



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- Bergstrom, D. E. and Lin, X., "Recent Advances in Palladium-Mediated Reactions of Nucleosides," *Nucleosides and Nucleotides* **1991**, *10*, 689-691.
- Bergstrom, D. E., Beal, P., Jenson, J., and Lin, X., "Palladium-Mediated Synthesis of C-5 Pyrimidine Nucleoside Thioethers from Disulfides and Mercurinucleosides," *J. Org. Chem.* **1991**, *56*, 5598-5602.
- Bergstrom, D. E. and Zhang, P. "An Efficient Route to C-4 Linked Imidazole Nucleosides: Synthesis of 2-Carbamoyl-4-(2'-deoxy-b-D-ribofuranosyl)imidazole," *Tetrahedron Letters* **1991**, *32*, 6485-6488
- Bergstrom, D., Lin, X., Wang, G., Rotstein, D., Beal, P., Norrix, K., and Ruth, J. "C-5 Substituted Nucleoside Analogs" *Synthetic Letters*. **1992**, 179-188.
- Bergstrom, D. and Schmaltz, T., "Organoiron-mediated Alkylation of Phosphite Esters: Synthesis of (Dicarbonyl)(h<sup>5</sup>-cyclopentadienyl)iron-Derived Nucleoside Phosphonate Esters," *J. Org. Chem.* **1992**, *57*, 873-876.
- Wang, G. and Bergstrom, D. E., "Controlled Oxidation of Dimethyl Derivatives of Pyridine, 2,2'-Bipyridine, and 1,10-Phenanthroline," *Synthetic Letters* **1992**, 422-425.
- Bergstrom, D. E., Mott, A. W., De Clercq, E., Balzarini, J., and Swartling, D. J., "3',3'-Difluoro-3'-deoxythymidine: Comparison of Anti-HIV Activity to 3'-Fluoro-3'-deoxythymidine," *J. Med. Chem.* **1992**, *35*, 3369-3372.
- Dalla Riva Toma, Joan, Toma, Pascal, Fanwick, P. E., Bergstrom, Donald E., Byrn, Stephen R. "Photochemical Synthesis and Crystal Structure of Two Potentially Useful Metal Carbonyl Complexes: Pentacarbonyl(m<sup>2</sup>-cis-cyclooctene)tungsten (0) and Tetracarbonylbis(m<sup>2</sup>-cis-cyclooctene)tungsten (0), *J. Crystallographic and Spectroscopic Research* **1993**, *23*, 41-47.
- Dalla Riva Toma, Joan, Toma, Pascal, Bergstrom, Donald E. "5-Chloromercurio-2'-deoxyuridine," *J. Crystallographic and Spectroscopic Research* in press **1993**.
- Wang, G., Bergstrom, D. E. "Synthesis of Oligonucleotides Containing N<sup>2</sup>-(5-Carboxypentyl)-2'-deoxyguanosine and 5-[2-(4'-Methyl-2,2'-dipyrid-4-yl-carboxamido)ethylthio]-2'-deoxyuridine," *Tetrahedron Letters*, in press **1993**, *34*, 6721-6724.
- Wang, G., Bergstrom, D. E. "Synthesis of Oligonucleotides Containing N<sup>2</sup>-[2-(Imidazol-4-ylacetamido)ethyl]-2'-deoxyguanosine," *Tetrahedron Letters*, in press **1993**, *34*, 6725-6728.

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME Michael J. BUNK	POSITION TITLE Director, Research resources Management
-------------------------	---

EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Iowa State University, Ames, IA	B.S.	1973	Zoology / Chemistry
Iowa State University, Ames, IA	M.S.	1976	Nutrition
Cornell University, Ithaca, NY	Ph.D.	1980	Nutritional Biochem.

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

1981-82 Postdoctoral Fellow, Monell Chemical Senses Ctr, Phila., PA.  
 1982-84 Postdoctoral Fellow, Sloan-Kettering Institute for Cancer Research, New York, NY.  
 1984-86 Research Associate, Memorial Sloan-Kettering Cancer Center (MSKCC), New York, NY.  
 1986-87 New Investigator, Clinical Nutrition Research Unit, MSKCC, New York, NY.  
 1986-89 Instructor in Nutrition/Medicine, Dept of Medicine, Cornell Univ. Med. College, New York, NY.  
 1987-89 Assistant Program Director, Clinical Nutrition Research Unit, MSKCC, New York, NY.  
 1989-91 Director of Foundation Relations, MSKCC, New York, NY.  
 1991-93 Senior Grants Management Specialist, MSKCC, New York, NY.  
 1993- Director, Research Resources Management, Strang Cornell Cancer Prevention Center, New York, NY.

*Honors and Awards:*

1977 American Egg Board Research Award.

*Representative Publications:*

Seres D, Bunk MJ, Osborne MP, Rivlin RS, Tiwari RK. (1991). Effects of marginal dietary zinc deficiency and vitamin E supplementation on hepatic ADPER activity in female Sprague-Dawley rats. *Nut. Res.* 11:337-346.

Bunk MJ, Dnistrian AM, Seres DA, Schwartz MK, Rivlin RS. (1990). Regulation of plasma concentrations of vitamin E by marginal intake of dietary zinc. *Proc. Soc. Exp. Biol. Med.*

Bunk MJ, Dnistrian AM, Schwartz MK, Rivlin RS. (1989). Dietary zinc deficiency decreased plasma concentrations of vitamin E. *Proc. Soc. Exp. Biol. Med.* 190:379-384.

Bunk MJ, Galvin J, Yung YP, Blaner WS. (1987). Relationship of cytotoxic activity of natural killer cells to growth rates and serum zinc levels of female R111 mice. *Nutr. and Cancer* 10:79-87.

Bunk MJ, Kinahan JJ, Sarkar NH. (1985). Biotransformation and protein binding of N-(4-hydroxyphenyl) retinamide in murine mammary epithelial cells. *Cancer Lett.* 26:319-326.

Bunk MJ, Telang NT, Traganos F, Sarkar NH. (1985). Effect of N-(4-hydroxyphenyl) retinamide on murine mammary tumor cells in culture. *Nutr. and Cancer* 7:23-32.

- Bunk MJ, Telang NT, Sarkar NH. (1983). Effect of malignant transformation upon the cellular retinoid binding proteins in cultured mammary tumor cells. *Cancer Lett.* 20:83-92.
- Bunk MJ, Combs FG Jr. (1981). Relationship of selenium-dependent glutathione peroxidase activity and nutritional pancreatic atrophy in selenium-deficient chicks. *J. Nutr.* 111:1611-1620.
- Bunk MJ, Combs GF Jr. (1981). Evidence for an impairment in the conversion of methionine to cysteine in the selenium-deficient chick. *Proc. Soc. Exp. Biol. Med.* 167:87-93.
- Bunk MJ, Combs GF Jr. (1980). Effect of selenium on appetite in the selenium-deficient chick. *J. Nutr.* 110:743-749.
- Bunk MJ, Combs FG Jr. (1979). Use of low viscosity epoxy plastic casts to study mammary core and mammary knob organization of avian eggshells. *Poultry Sci.* 58:1340-1344.
- Bunk MJ, Balloun SL. (1977). Ultrastructure of the mammary region of poor quality avian eggshells. *Poultry Sci.* 57:639-647.
- Bunk MJ, Balloun SL. (1977). Structure and relationship of the mammary core to membrane fibers and initial calcification of the avian eggshell. *Br. J. Poultry Sci.* 18:617-621.

#### CHAPTERS AND REVIEWS:

- Bunk MJ, Schloen L., Rivlin RS. Nutrition and Cancer. (1988). In *Manual of Clinical Nutrition*. DM Paige and TM Bayless, eds. (Washington D.C., Nutrition Publications, Inc. ), PP392-407.
- Bunk MJ, Rivlin RS. (1987). Nutrition and Prevention of Cancer: Some Comments on Recent Developments. In *Current Concepts* 6(1):1-7.

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME Ronald M. COOK	POSITION TITLE President/Chief Technical Officer
------------------------	---

EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
University of California, Berkeley, Coll. of	B.S.	1969	Chemistry
University of Washington, Seattle	Ph.D.	1974	Chemistry
University of California, S.F., Med School	Postdoc.	74-76	Immun/Microbiology
University of California, S.F., Med School	Rsh.Assoc	1977	Biochem./Biophy

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

**University of California, San Francisco, (UCSF), Med School, Post Doc, Dept of Microbiology and Immunology, 1974-76--** Investigations related to determining the molecular parameters leading to immune response, particularly the antigen structural requirements for lymphocyte activation.

**University of California, San Francisco, (UCSF), Med School, Research Associate, Dept of Biochemistry and Biophysics, 1976-77.** Synthesis of collagen like peptides for investigation of structural qualities of mammalian dermal tissue.

**Biosearch, a Sole Proprietorship-1977-1981. Founder and President**

Products: Specialty BioChemicals for Immunology and Neuropharmacological Research. Synthetic Peptides and small molecule haptens and conjugates.

**Biosearch, Inc-1981-1989. Chairman of the Board, Director of Research/ Development and Engineering. Responsible for all product development of Biosearch** Revenues of ca \$15 million in 1988, about \$10 Million in instrumentation and \$5 Million in reagents. Directed R,D and E staff of 25 scientists and engineers with annual budget of \$2.5 million.

Chemistries utilized included solution phase triester, solid phase triester (MSNT and MsCl/N-methyl imidazole), amidite, and H-phosphonate. Biosearch was first to demonstrate the superiority of cyanoethyl amidites by the routine synthesis of oligomers larger than 100 residues. Instrumentation included: SAM I DNA Synthesizer (1982)--First commercially successful DNA synthesizer; 8600, 8700 DNA Synthesizers (1986, 1987)--Currently top line instruments for Milligen Biosearch; 8800 Preparative Scale DNA Synthesizer--for antisense DNA research and preparation of bulk diagnostic probes; Cyclone(1987)--popular low cost DNA Synthesizer.

Biosearch was first to commercialize FMOC automated chemistries (1984), and demonstrated BOP/HOBt as a superior coupling method to symmetric anhydrides. Instrumentation included: SAM II Peptide Synthesizer; 9500 Peptide Synthesizer; Excel Peptide Synthesizer-

Other technologies developed at Biosearch included: Automated DNA Sequencing via CCD Camera Detection of Fluorescent DNA; Development of Advanced Instrumentation for Protein Sequencing; RNA Synthesis via 2' Silyl Protection; Biopolymer purification via covalent cleavable affinity labels; DNA sequencing via Mass labels.

**1990-Present Siris Laboratories: President/Chief Technical Officer**

SIRIS Labs engages in research and development of advanced systems and chemistries for the application of biopolymers to biotechnology problems. The generation and application of peptide and DNA

arrays are central to the focus of Siris' business. Over the past two years Siris has acted as principal consultant to Beckman Instrument's (Fullerton, Ca) Advanced Development Unit for the development of Beckman's DNA synthesizer, the OLIGO 1000 (Released to Market August 1992). Further contributions have centered on novel DNA amplification technology applicable to DNA diagnostics for which a patent has been applied.

**Patents:** DNA Synthesis and Peptide Chemistry and Instrumentation; several issued and in application

**Grants:** Several SBIR's in 1987-93 on peptide and DNA chemistry.

**Representative Publications:**

- A. Ali, RM Cook, B Weinstein, Amino Acids and Peptides-Synthesis of an Undecapeptide Sequence (A43-A53) of Rubredoxin, *Int Journal of Peptide and Protein Research*, Vol 4, 144-180(1972).
- D. Stevenson, RM Cook, B Weinstein, Synthesis of a Nonapeptide Sequence A11-A19) of Rubredoxin, *Int Journal of Peptide and Protein Research*, Vol 4, 101-108(1972).
- RM Cook, D. Stevenson, B. Weinstein, Synthesis of a Decapeptide Sequence (A28-A37) of Rubredoxin, Amino Acids and Peptides XXXVI, *Int Journal of Peptide and Protein Research*, Vol 6, 55-58(1974).
- RM Cook, B Weinstein, R. Tam, The Synthesis of a Peptide Having the Structure Attributed to a Sound Habituating Material, *Experientia* 31,754 (1975).
- RM Cook, S. Fong, DE Nitecki, JW Goodman, Spacial Requirements Between Haptenic and Carrier Determinants for T-Dependent Antibody Responses, *Journal of Experimental Medicine*, 78, 817, 1976.
- LC Altman, E. Shiffman, and RM Cook, The Preparation of Formyl-Met-Leu-Phe-silver Sulfadiazine, A compound with Leukoattractant and Antimicrobial Activity, FASEB, 1980.
- RM Cook and D. Hudson, Solid Phase Nucleotide and Peptide Synthesis By Adaptation Of Laboratory HPLC Equipment, *Proceedings of the Miami Winter Symposium on Gene Expression*, 1982.
- RM Cook, et al, Principles of Automated Gene Fragment Synthesis, in *Chemical and Enzymatic Synthesis of Gene Fragments*, HG Gassen, Ed., Verlag-Chemie, 1982.
- RM Cook, Instrumentation for Automated Gene Fragment Synthesis, in *Biotechnology*, Paul Cheremisinoff, Ed., Technomic, 1985.
- RM Cook, "DNA Synthesizers Provide 'Catalyst' for Genetic Research Gains", *R and D Magazine*, May 1984.
- D. Hudson, RM Cook, et al, FMOC Mediated Solid Phase Assembly of HIV TAT Protein, *Proceedings of the 20th European Peptide Symposium*, Gunther Jung/Ernst Bayer, Eds., de Gruyter(Berlin), 1989.
- M. Lyttle, RM Cook, P. Wright, Large Scale Automated DNA Synthesis, *Biopharm Manufacturing*, July/August, 1988
- ND Sinha and RM Cook, The Preparation and application of functionalized synthetic oligonucleotides: III Use of phosphonate derivatives of protected amino hexanol and mercapto propanol or hexanol, *Nucleic Acids Research*, 16(6), 2659, IRL Press, 1988.
- RM Cook, S. Biancalana, D. Hudson, AD Frankel, DS Tsou, M. Lyttle, and N. Sinha, Approaches Towards the Automated Synthesis of Genes and Proteins, paper presented at the conference, Innovation and Perspectives in Solid Phase Synthesis, Oxford University, England, Sept, 1989.

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME Melissa A. COTHERN		POSITION TITLE Graduate Assistant	
EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
University of Florida, FL	B.S.	1993	Chemistry

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

Undergraduate Research with Professor Eric Enholm at the University of Florida. Ms. Cothorn has extensive experience in performing organic reactions including those requiring techniques such as exclusion of moisture and air.

*Honors and Awards:*

Florida Undergraduate Scholarship  
Undergraduate Honor Society

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME James M. COULL		POSITION TITLE Specialty Chemistry Group Manager	
EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Colby College, Waterville, ME	B.A.	1980	Chemistry/Biochemistry
Purdue University, West Lafayette, IN	Ph.D.	1986	Biochemistry

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

1987-1990 Millipore Corporation, Research Chemist  
1990-Present Millipore Corporation, Specialty Chemistry Group Manager

*Honors and Awards:*

Arnold Kent Award for Outstanding Scholarship and Research as a graduate student  
Author and Co-recipient of an American Cancer Society competitive grant while a graduate student  
NIH Predoctoral Traineeship, July 1983 to December 1986  
Millipore Technical Innovation Award for development of Sequelon membranes for protein and peptide analysis

*Representative Publications:*

- Toren, P.C., Betsch, D.F., Weith, H.L. and Coull, J.M. (1986) Determination of Impurities in Nucleoside 3'-Phosphoramidites by Fast Atom Bombardment Mass Spectrometry. *Analytical Biochemistry* 152:291-294.
- Coull, J.M., Weith, H.L. and Bischoff, R. (1986) A Novel Method for the Introduction of an Aliphatic Primary Amino Group at the 5' Terminus of Synthetic Oligonucleotides. *Tetrahedron Letters* 27:3991-3994.
- Bischoff, R., Coull, J.M. and Regnier, F.E (1987) Introduction of 5'-Terminal Functional Groups into Synthetic Oligonucleotides for Selective Immobilization. *Analytical Biochemistry* 164:336-344.
- Coull, J.M., Carlson, D.V. and Weith, H.L. (1987) Synthesis and Characterization of a Carbamate-Linked Oligonucleoside. *Tetrahedron Letters*, 28:745-748.
- Beck, S., O'Keeffe, T., Coull, J.M. and Köster, H. (1989) Chemiluminescent Detection of DNA: Application for DNA Sequencing and Hybridization. *Nucleic Acids Research* 17:5115-5123.
- Coull, J.M., Dixon, J.D., Laursen, R.A., Köster, H. and Pappin, D.J.C. (1989) Development of Membrane Supports for the Solid-phase Sequence Analysis of Proteins and Peptides. In *Methods in Protein Sequence Analysis* (B. Wittman-Liebold, Ed.) pp. 69-78, Springer-Verlag, Berlin.
- Daniels, S.B., Bernatowicz, M.S., Coull, J.M. and Köster, H. (1989) Membranes as Solid Supports for Peptide Synthesis. *Tetrahedron Letters* 30:4345-4348.

- Pappin, D.J.C., Coull, J.M. and Köster, H. (1990) Solid-Phase Sequence Analysis of Proteins Electroblotted or Spotted onto Polyvinylidene Difluoride Membranes. *Analytical Biochemistry* 187:10-19.
- Bernatowicz, M.S., Daniels, S.B., Coull, J.M., Kearney, T., Neves, R.S., Coassin, P.J. and Köster, H. (1990) Recent Developments in Solid Phase Peptide Synthesis using the 9-Fluorenylmethoxycarbonyl (Fmoc) Protecting Group Strategy. In *Current Research in Protein Chemistry: Techniques, Structure, and Function* (J. Villafranca, ed.) pp. 63-77, Academic Press, San Diego.
- Pappin, D.J.C., Coull, J.C., and Köster, H. (1990) New Approaches to Covalent Sequence Analysis. In *Current Research in Protein Chemistry: Techniques, Structure, and Function* (J. Villafranca, ed.) pp. 191-202, Academic Press, San Diego.
- Gildea, B.D., Coull, J.M. and Köster, H. (1990) A Versatile Acid-Labile Linker for Modification of Synthetic Biomolecules. *Tetrahedron Letters* 31:7095-7098.
- Coull, J. M., Pappin, D.J.C., Mark, J., Aebersold, R. and Köster, H. (1991) Functionalized Membrane Supports for Covalent Protein Microsequence Analysis. *Analytical Biochemistry* 194:110-120.
- Bodwell, J.E., Orti, E., Coull, J.M., Pappin, D.J.C., Smith, L.I., and Swift, F. (1991) Identification of Phosphorylated Sites in the Mouse Glucocorticoid Receptor. *J. Biol. Chem.* 266:7549-7555.



**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME Patrice COURVALIN	POSITION TITLE Professor
---------------------------	-----------------------------

EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Institut Pasteur, France	M.S.	1971	Microbiology
Institut Pasteur, France	M.S.	1972	Human Biology
Institut Pasteur, France	M.D.	1974	Medical Sciences

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

**Professional Experience:**

1970-1973	Medical Bacteriology Unit, Research Associate, Prof. Y.A. CHABBERT, Department of Bacteriology and Mycology, Institut Pasteur.
1973-1974	Oncogenic Viruses Unit, Research Associate, Prof. F. CUZIN, Department of Molecular Biology, Institut Pasteur.
1974-1977	Research Associate, Laboratory of Prof. J.E. DAVIES, Department of Biochemistry, University of Wisconsin, Madison, USA.
1977-1983	Laboratory of Biochemistry, Medical Bacteriology Unit, Assistant Professor, with Prof. Y.A. CHABBERT, Department of Bacteriology and Mycology, Institut Pasteur.
1983-1989	Antibacterial Agents Unit, CNRS U.A. 271, Associate Professor, Department of Bacteriology and Mycology, Institut Pasteur.
1989-1990	Visiting Scholar, Laboratory of Prof. D.R. HELINSKI, Center for Molecular Genetics, University of California, San Diego, USA.
1990-present	Professor, Antibacterial Agents Unit, Department of Bacteriology and Mycology, Institut Pasteur.

**Honors and Awards:**

1974	Paris School of Medicine award.
1983	Thérèse Lebrasseur award of the Fondation de France.
1989	Jacques Monod award of the Fondation de France.

**Representative Publications:**

- Courvalin, P., C. Carlier, and Y. A. Chabbert. 1972. Plasmid-linked tetracycline and erythromycin resistance in group D "*Streptococcus*". *Ann. Inst. Pasteur* **123** : 755-759.
- Courvalin, P., C. Carlier, O. Croissant, and D. Blangy. 1974. Identification of two plasmids determining resistance to tetracycline and to erythromycin in group D *Streptococcus*. *Mol. Gen. Genet.* **132** : 181-192.
- Courvalin, P., B. Weisblum, and J. Davies. 1977. Aminoglycoside-modifying enzyme of an antibiotic-producing bacterium acts as a determinant of antibiotic resistance in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **74** : 999-1003.
- Courvalin, P., and M. Fianndt. 1980. Aminoglycoside-modifying enzymes of *Staphylococcus aureus* : Expression in *Escherichia coli*. *Gene* **9** : 247-269.

Labigne-Roussel, A., G. Gerbaud, and P. Courvalin. 1981. Translocation of sequences encoding antibiotic

- resistance from the chromosome to a receptor plasmid in *Salmonella ordonez*. *Mol. Gen. Genet.* **182** : 390-408.
- Labigne-Roussel, A., J.L. Witchitz, and P. Courvalin. 1982. Modular evolution of disseminated Inc 7-M plasmids encoding gentamicin resistance. *Plasmid* **8** : 215-231.
- Labigne-Roussel, A., and P. Courvalin. 1983. IS15, a new insertion sequence widely spread in R plasmids of Gram-negative bacteria. *Mol. Gen. Genet.* **189** : 102-112.
- Trieu-Cuot, P., G. Gerbaud, T. Lambert, and P. Courvalin. 1985. *In vivo* transfer of genetic information between Gram-positive and Gram-negative bacteria. *EMBO J.* **4** : 3583-3587.
- Arthur, M., and P. Courvalin. 1986. Contribution of two different mechanisms to erythromycin resistance in *Escherichia coli*. *Antimicrob. Agents Chemother.* **30** : 694-700.
- Courvalin, P., and C. Carlier. 1986. Transposable multiple antibiotic resistance in *Streptococcus pneumoniae*. *Mol. Gen. Genet.* **205** : 291-297.
- Courvalin, P., and C. Carlier. 1987. Tn1545 : A conjugative shuttle transposon. *Mol. Gen. Genet.* **206** : 259-264.
- Brisson-Noël, A., M. Arthur, and P. Courvalin. 1988. Evidence for natural gene transfer from Gram-positive cocci to *Escherichia coli*. *J. Bacteriol.* **170** : 1739-1745.
- Leclercq, R., E. Derlot, J. Duval, and P. Courvalin. 1988. Plasmid-mediated resistance to vancomycin and teicoplanin in *Enterococcus faecium*. *N. Engl. J. Med.* **319** : 157-161.
- Poyart-Salmeron, C., P. Trieu-Cuot, C. Carlier, and P. Courvalin. 1989. Molecular characterization of two proteins involved in the excision of the pneumococcal transposon Tn1545 : homologies with other site-specific recombinases. *EMBO J.* **8** : 2425-2433.
- Dutka-Malen, S., C. Molinas, M. Arthur, and P. Courvalin. 1990. The VANA glycopeptide resistance protein is related to D-alanyl-D-alanine ligase cell wall biosynthesis enzymes. *Mol. Gen. Genet.* **224** : 364-372.
- Arthur, M., C. Molinas, C. Mabilat, and P. Courvalin. 1990. Detection of erythromycin resistance by the polymerase chain reaction using primers in conserved regions of *erm* rRNA methylase genes. *Antimicrob. Agents Chemother.* **34** : 2024-2026.
- Poyart-Salmeron, C., P. Trieu-Cuot, C. Carlier, and P. Courvalin. 1990. The integration-excision system of the streptococcal transposon Tn1545 is structurally and functionally related to those of lambdoid phages. *Mol. Microbiol.* **4** : 1513-1521.
- Mabilat, C., and P. Courvalin. 1990. Development of "Oligotyping" for characterization and molecular epidemiology of TEM  $\beta$ -lactamases in *Enterobacteriaceae*. *Antimicrob. Agents Chemother.* **34** : 2210-2216.
- Arthur, M., C. Molinas, and P. Courvalin. 1992. The VanS-VanR two-component regulatory system controls synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. *J. Bacteriol.* **174** : 2582-2591.
- Arthur, M., C. Molinas, F. Depardieu, and P. Courvalin. 1993. Characterization of Tn1546, a Tn3-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. *J. Bacteriol.* **175** : 117-127.

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME Darren DAY	POSITION TITLE Postdoctoral Research Fellow
--------------------	--

EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
University of Southampton, Southampton, U.K.	BSc. Hons	1986	Biochemistry
University of Southampton, Southampton, U.K.	Ph.D.	1989	Biochemistry
University of Southampton, Southampton, U.K.	Postdoc.	1990	Biochemistry
Auckland University, Auckland, New Zealand.	Postdoc	90-92	Molecular Biology

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

1989-1990 University of Southampton, Postdoctoral Fellow; C. Anthony.  
 1990-1992 Auckland University, Postdoctoral Fellow; P.L. Bergquist.  
 1992-present Cornell University Medical College, Postdoctoral Fellow, F. Barany.

*Representative Publications:*

- Nunn, D.N., Day, D.J. and Anthony, C. (1989). The second subunit of methanol dehydrogenase of *Methylobacterium extorquens* AM1. *Biochem. J.*, 260:857-862.
- Day, D.J., Nunn, D.N. and Anthony, C. (1990). Characterisation of a novel *c*-type cyochrome in a *moxD* mutant in *Methylobacterium extorquens* AM1. *J. Gen. Microbiol.*, 136:181-188.
- Day, D.J. and Anthony, C. (1990). Methanol dehydrogenase from *Methylobacterium extorquens* AM1. *Meth. Enzymol.*, 188:210-216.
- Day, D.J. and Anthony, C. (1990). Soluble cytochromes *c* of methanol-utilising bacteria. *Meth. Enzymol.*, 188:298-303.
- Cox, J., Day, D.J. and Anthony (1991). The interaction of methanol dehydrogenase and its electron acceptor, cytochrome *c<sub>L</sub>* in methylotrophic bacteria. *Biochem. Biophys. Acta*, 119:97-106.
- Day, D.J., Saul, D.J., Reeves, R.A. and Bergquist, P.L. (1993). A solid phase assay for thermophilic DNA polymerases. *Anal. Biochem.*, 211:174-176.

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME Jack FISHMAN	POSITION TITLE Director of Research
----------------------	--

EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Yeshiva University, New York, NY	B.A.	1950	Chemistry
Columbia University, New York, NY	M.A.	1952	Chemistry
Wayne University, Detroit, MI	Ph.D.	1955	Chemistry
Oxford University, UK	Fellow	55-56	Chemistry

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

1956-1959	Research Associate, Div. of Steroid Metabolism and Biochemistry, Sloan-Kettering Institute, NY
1959-1960	Assistant Member, Sloan-Kettering Institute, New York
1960-1963	Associate, Member, Sloan-Kettering Institute, New York
1963-1970	Investigator, Institute for Steroid Research, Montefiore Hospital, Bronx, NY
1967-1970	Associate Professor, Albert Einstein College of Medicine, Bronx, NY
1970-1974	Senior Investigator, Institute for Steroid Research, Montefiore Hospital, Bronx, NY
1971-1980	Professor, Albert Einstein College of Medicine, Bronx, NY
1974-1977	Director, Institute for Steroid Research, Montefiore Hospital, Bronx, NY
1977-1980	Adjunct Professor, The Rockefeller University, New York, NY
1980-1988	Professor, The Rockefeller University, New York, NY
1988-	Adjunct Professor, The Rockefeller University, New York, NY
1988-1991	President, IVAX Corporation
1989-1991	Research Professor, University of Miami, Miami, FL
1991-	Professor of Biochemistry, Cornell University Medical College, New York, NY
1991-	Director of Research, Strang-Cornell Cancer Research Laboratory, New York, NY

*Honors and Awards:*

1976	U.S. Steroid Chemistry and Biochemistry Delegation to the Peoples Republic of China
1977-1981	Endocrinology Study Section, National Institutes of Health, Bethesda, MD
1981-1982	Chairman, Scientific Affairs Committee, The Endocrine Society
1982	John Scott Medal Award - For the development of naloxone
1982-1984	Chairman, Endocrinology Study Section, National Institutes of Health, Bethesda, MD
1992	Honorary D.Sc. Yeshiva University, NY.

*Representative Publications:*

- Miyairi, S., and Fishman, J. Radiometric analysis of oxidative reactions in aromatization by placental microsomes. Presence of differential isotope effects. *J.Biol.Chem.* 260:320-325, 1985.
- Miyairi, S., Sugita, O., Sassa, S. and Fishman, J. Aromatization and 19-hydroxylation of androgens by rat brain cytochrome P-450. *Biochem. Biophys. Res. Commun.* 150: 311-315, 1988.

- Lustig, R.H., Pfaff, D.W. and Fishman, J. Opioidergic modulation of the oestradiol-induced LH surge in the rat: roles of ovarian steroids. *J. Endocrinol.* 116: 59-69, 1988.
- Michnovicz, J.J., Hershcopf, R.J., Naganuma, H., Bradlow, H.L. and Fishman, J. Increased 2-hydroxylation of estradiol as a possible mechanism for the anti-estrogenic effect of cigarette smoking. *New Engl. J. Med.* 315:1305-1309, 1986.
- Bradlow, H.L., Hershcopf, R.J. and Fishman, J. Oestradiol 16 $\mu$ -hydroxylase: a risk marker for breast cancer. *Cancer Surveys*, 5: 573-583, 1986.
- Sugita, O., Sassa, S., Miyairi, S., Fishman, J., Kubota, I., Noguchi, T. and Kappas, A. Cytochrome P-450 c-M/F a new constitutive form of microsomal cytochrome P-450 in male and female rat liver with estrogen 2- and 16 $\mu$ -hydroxylase activity. *Biochemistry*, 27: 678-686, 1988.
- Fishman, J.H. and Fishman, J. Distinction between malignant and normal breast tissue based on endogenous mediators of estradiol binding. *Biochem. Biophys. Res. Commun.* 150: 1131-1137, 1988.
- Norton, B.I. Miyairi, S. and Fishman, J. 19-Hydroxylation of androgens by rat granulosa cells. *Endocrinology*, 122: 1047-1052, 1988.
- Swaneck, G.E. and Fishman, J. Covalent binding of the endogenous estrogen 16 $\mu$ -hydroxyestrone to estradiol receptor in human breast cancer cells: characterization and intranuclear localization. *Proc. Natl. Acad. Sci. USA*, 85: 7831-7835, 1988.
- Jellinck, P.H. and Fishman, J. Activation and irreversible binding of regiospecifically labelled catechol estrogen by rat liver microsomes: Evidence for differential cytochrome P-450 catalyzed oxidations. *Biochemistry*, 27: 6111-6116, 1988.
- Michnovicz, J.J., Naganuma, H., Hershcopf, R.J., Bradlow, H.L. and Fishman, J. Increased urinary catecholesterogen excretion in female smokers. *Steroids*, 52: 69-83, 1988.
- Lustig, R., Mobbs, C.V., Pfaff, D.W. and Fishman, J. Temporal actions of 16 $\mu$ -hydroxyestrone in the rat: comparisons of lordosis dynamics with other estrogen metabolites and between sexes. *J. Steroid Biochem.* 33: 417-421, 1989.
- Naganuma, H., Hershcopf, R.J., Michnovicz, J.J., Miyairi, S., Bradlow, H.L. and Fishman, J. Radioimmunoassay of 16 $\mu$ -hydroxyestrone in human urine. *Steroids*, 53: 37-48, 1989.
- Niwa, T., Bradlow, H.L., Fishman, J. and Swaneck, G.E. Determination of estradiol 2- and 16-alpha-hydroxylase activities in MCF-7 human breast cancer cells in culture using radiometric analysis. *J. Steroid Biochem.* 22: 311-314, 1989.
- Lustig, R.H., Mobbs, C.V., Bradlow, H.L., McEwen, B.S. and Fishman, J. Differential effects of estradiol and 16 $\mu$ -hydroxyestrone on pituitary and pre-optic estrogen receptor regulation. *Endocrinology*, 125:2701-2709, 1989.
- Swaneck, G.E. and Fishman, J. Effects of estrogens on MCF-7 cells: positive or negative regulation by the nature of the ligand-receptor complex. *Biochem. Biophys. Res. Commun.* 174: 276-281, 1991.
- Swaneck, G.E. and Fishman, J. Estrogen actions on target cells: evidence for different effects by products of two alternative pathways of estradiol metabolism. In: *The New Biology of Steroid Hormones*, edited by R.B. Hochberg and F. Naftolin, Raven Press, New York, 1991, pp. 47-70.
- Martucci, C.P. and Fishman, J. P-450 Enzymes of estrogen metabolism. *Pharmac. Ther.* 57:237-257, 1993.

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME Wilbur A. FRANKLIN		POSITION TITLE Professor	
EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
University of Colorado, Boulder, CO	B.A.	1964	
Northwestern University Medical School	M.D.	1968	Medicine
Northwestern University Medical School, Intern & Res	Board Cert.	1968-72	Anatomic & Clinical Pathology

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

**Professional Experience:**

1972-73 Assistant Professor of Pathology, Blood Bank, George Washington University Medical School  
 1973-74 Assistant Professor of Pathology, Georgetown University Medical School, Washington, D.C.  
 1972-74 Staff Pathologist, National Naval Medical Center, Bethesda, MD  
 1974-76 Post-doctoral Fellow, Immunology, LaRabida-University of Chicago Institute  
 1976-83 Assistant Professor, Surgical Pathology, University of Chicago  
 1983-84 Visiting Research Associate, John Radcliffe Hospital, Oxford, England  
 1984-89 Associate Professor, Surgical Pathology, University of Chicago  
 1989- Professor and Chief, Surgical Pathology, University of Colorado and VA Hospital

**Military Service:**

1972-73 U.S. Navy, Department of Pathology, National Naval Medical Center, Bethesda, MD

**Societies/Honors:**

American Association of Pathologists, American Association for Cancer Research, International Academy of Pathology, Author Purdy Stout Society, Cell Kinetics Society, National Association for the Advancement of Science, New York Academy of Science, Phi Beta Kappa Junior Faculty Clinical Fellow, American Cancer Society, 1979-1982

**Selected Publications (from a total of 61):**

Franklin, W.A., Falini, B., Pulford, K.A.F., Clarke, L., Stein, H., O'D McGee, J., Bliss, E.A., Gatter, K.C., Mason, D.Y. (1986) Immunohistochemical analysis of human mononuclear phagocytes by using monoclonal antibodies. *Lab. Invest.* **54**: 322-335.

Kadin, M.E., Schweid, A., Ireland, K., Franklin, W.A., Dorfman, R.F. (1986) Ki-1 lymphoma in childhood. *Blood* **68**: 1042-1049.

Franklin, W.A., Hogg, N., Mason, D.Y. (1987) Human leucocyte differentiation antigens: Review of the Third International Workshop. *Mol. Cell. Probes* **1**: 55-60.

McGurrin, J., Doria, M.I., Dawson, P.J., Karrison, T., Stein, H., Franklin, W.A. (1987) Assessment of tumor cell kinetics by immunohistochemistry in carcinoma of breast. *Cancer* **59**: 1744-1750.

Fisfalen, M.E., De Groot, L.J., Quintans, J., Franklin, W.A., Soltani, K. (1988) Microsomal antigen reactive lymphocyte lines and clones derived from thyroid tissue of patients with Grave's disease. *J. Endocrin. Metab.* **66**: 776-784.

- Portmann, L., Fitch, F.W., Havran, W., Hamada, N., Franklin, W.A., De Groot, L.J. (1988) Characterization of the thyroid microsomal antigen, and its relationship to thyroid peroxidase, using monoclonal antibodies. *J. Clin. Invest.* **81**: 1217-1224.
- Montag, A.G., Geradts, J., Hui, P.K., Doria, M.I., Franklin, W.A. (1988) Limited expression of myeloid antigens by neuroendocrine tumors of lung. In: Souhami, R.L., Bobrow, L.G., Beverley, P.C.L., eds.; Proceedings of the first international workshop on small cell lung cancer antigens. *Lung Cancer* **4**: 55-57.
- Doria, M.I., Franklin, W.A. (1988) Immunophenotype of small cell lung carcinoma: Expression of NKH-1 and transferrin receptor and absence of most myeloid antigens. *Cancer* **62**: 1939-1945.
- Park, J.K., McKeithan, T.W., Le Beau, M.M., Bitter, M.A., Franklin, W.A., Rowley, J.D., Diaz, M.O. (1989) An (8;14) (q24;q11) translocation involving the T-cell receptor alpha-chain and the myc oncogene 3' region in a B-cell lymphoma. *Genes, Chromosomes & Cancer* **1**: 15-22.
- Colley, M.H., Kossmoss, F., Bibbo, M., Dytch, H.E., Holt, J.A., Wied, G.L., Franklin, W.A. (1989) Assessment of hormone receptor in breast carcinoma by immunocytochemistry and image analysis. II. Estrogen receptors. *Anal. Quant. Cytol. Hist.* **11**: 307-314.
- Le Beau, M.M., Bitter, M.A., Larson, R.A., Doane, L.A., Franklin, W.A., Rubin, C.M., Vardiman, J.W. (1989) The t(2;5) (p23;q35): A recurring chromosomal abnormality in Ki-1 positive non-Hodgkin's lymphoma. *Leukemia* **3**: 866-870.
- Bitter, M.A., Le Beau, M.M., Franklin, W.A., Larson, R.A., McKeithan, T.W., Rubin, C.M., Stephens, J.K., Vardiman, J.W. (1990) Morphology in Ki-1 (CD30)-positive non-Hodgkin's lymphoma is correlated with clinical features and the presence of a unique chromosomal abnormality, t(2;5) p23;q35). *Am. J. Surgical Path.* **14**: 305-316.
- Colley, M., Kossmoss, F., Franklin, W.A. (1990) In situ distribution of c-erbB2 protein, EGF receptor and PDGF receptor in carcinoma of breast. *Mol. Cell. Probes* **4**: 11-23.
- Franklin, W.A., Christison, W.H., Colley, M., Montag, A.G., Stephens, J.K., Hart, C.E. (1990) In situ distribution of the beta subunit of PDGF factor receptor in non-neoplastic tissue and in soft tissue tumors. *Cancer Res.* **50**: 6344-6348.
- Franklin, W.A. (1992) Retinoids: Old data and new. *Lung Cancer Res. Quarterly* **2**: 10-20.
- Shroyer, K.R., Kim, J.G., Greer, C.E., Manos, M., Pearlman, N.W., Franklin, W.A. (1992) Papillomavirus DNA in anorectal squamous carcinoma but not in colonic adenocarcinoma. *Arch. Surgery* **127**: 741-774.
- Franklin, W.A.: Immunophenotypic changes associated with malignant transformation of respiratory tract epithelium. *Lung Cancer* (in press).
- Shpall, E.J., Jones, R.B., Franklin, W.A., Curiel, T., Bitter, M.A., Claman, H., Archer, P., Bearman, S.I., Stemmer, S.M., Myers, S., Hami, L., Johnston, C., Taffs, S., Heimfeld, S., Hallagan, J., Berenson, R.J.: Transplantation of autologous CD34+ hematopoietic progenitor cells into breast cancer patients following high-dose chemotherapy: Influence of cell source and growth factors on engraftment. *J. Clin. Oncol.* (in press).
- Franklin, W.A., Kennedy, T.C., Miller, Y., Meyer, A., Folkvoord, J., Garza-Williams, S., Parks, T., Bunn, P.A.: Expression of epidermal growth factor receptor, neural cell adhesion molecule, Ki-67 and transferrin receptor by dysplastic bronchial epithelium. *Int. J. Cancer* (In Press).

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME Stephen H. FRIEND		POSITION TITLE Assistant Professor of Pediatrics	
EDUCATION <i>(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)</i>			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Indiana University, Bloomington, IN	B.A.	1975	Philosophy
Indiana University, Bloomington, IN	Ph. D.	1979	Chemistry
Indiana University, School of Medicine, IN	M.D.	1981	Medicine

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

1981-1982	Intern in Pediatrics, Children's Hospital of Philadelphia
1982-1984	Resident in Pediatrics, Children's Hospital of Philadelphia
1984-1985	Clinical Fellow in Hematology/Oncology - The Children's Hospital Boston, MA
1985-1987	Research Fellow, Harvard Medical School, Boston, MA
1985-1989	Visiting Scientist, Whitehead Institute for Medical Research, Cambridge, MA
1987-1988	Instructor in Pediatrics, Harvard Medical School, Boston, MA
1988-present	Assistant Professor of Pediatrics, Harvard Medical School, Massachusetts General Hospital, Boston, MA
1989-1990	Assistant Member, MGH Cancer Center, Massachusetts General Hospital, Boston, MA
1990-1991	Associate Member, MGH Cancer Center, Massachusetts General Hospital, Boston, MA
1990-present	Faculty Member, Cell and Developmental Biology Program, Harvard Medical School, Boston, MA
1991- present	Member, MGH Cancer Center

*Honors and Awards:*

1975	Phi Beta Kappa
1987	Lucille P. Markey Trust Scholar
1990	Merck Foundation Research Award
1991	Sun Life of Canada Award
1992	American Society for Clinical Investigation
1993	General Motors Visiting Professor: National Cancer Institute

*Representative Publications:*

Friend SH, Bernards R, Rogelj S, Weinberg RA, Rappaport JM, Albert DM, Dryja TP. Identification of a human DNA segment having properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature* 1986; 323:64.

Friend SH, Horowitz JM, Gerber MR, Wang X-FW, Bogenmann E, Li FP, Weinberg RA. Deletions of a DNA sequence in retinoblastomas and mesenchymal tumors: Organization of the sequence and its encoded protein. *Proc Natl Acad Sci, USA* 1987; 84:9059-9063.



- Friend SH, Dryja TP, Weinberg RA. Oncogenes and tumor-suppressing genes. *N Engl J Med* 1988; 318:618-622.
- Whyte P, Buchkovich KJ, Horowitz JM, Friend SH, Raybuck M, Weinberg RA, Harlow E. Association between an oncogene and an anti-oncogene: The adenovirus E1a proteins bind to the retinoblastoma gene product. *Nature* 1988; 334:124-129.
- Bernards R, Schackleford GM, Gerber MR, Horowitz JM, Friend SH, Scharl M, Bogenmann E, Rappaport JM, McGee T, Dryja TP, Weinberg RA. Structure and expression of the murine retinoblastoma gene and characterization of its encoded protein. *Proc Natl Acad Sci USA* 1989; 86:6474-6478.
- Diller L, Kassel J, Nelson CE, Gryka M, Litwak G, Gebhardt M, Bressac B, Ozturk M, Baker SJ, Vogelstein B, Friend SH. p53 Functions as a cell cycle control protein in osteosarcomas. *Mol and Cell Biol* 1990; 10:5772-5781.
- Malkin D, Li FB, Strong LC, Fraumeni, Jr JF, Nelson CE, Kim DH, Kassel J, Gryka MA, Bischoff FZ, Tainsky MA, Friend SH. Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. *Science* 1990;250:1233-1238.
- Børresen AL, Hovig E, Smith-Sorensen B, Malkin D, Sigrid L, Andersen TI, Nesland J, Isselbacher KJ, Friend SH. Constant denaturant gel electrophoresis as a rapid screening technique for p53 mutations. *Proc Natl Acad Sci USA* 1991; 88:8409.
- Maklin D, Jolly KW, Barbier N, Look AT, Friend SH, Gebhardt MC, Andersen TI, Børresen A-L, Li F, Garber J, Strong LC. Germline mutations of the p53 tumor suppressor gene in children and young adults with second malignant neoplasms. *N Engl J Med* 1992;326:1309-1315.
- Børresen A-L, Andersen Ti, Garber J, Piraux N-B, Thorlacius S, Eyfjord J, Ottestad L, Sorensen B-S, Pharm M, Hovig E, Malkin D, Friend SH. Screening for germ-line p53 mutations in breast cancer patients. *Cancer Research* 1992; 52:3234-3236.
- Frebourg T, Kassel J, Lam KT, Gryka MA, Piraux N-B, Andersen TI, Børresen A-L, Friend, SH. Germline mutations of the p53 tumor suppressor gene in patients with high risk for cancer inactivate the p53 protein. *Proc Natl Acad Sci USA* 1992; 89:6413-6417.
- Destree O, Lam K, Peterson-Maduro LJ, Eizema K, Diller L, Gryka M, Frebourg T, Shibuya E, Friend SH. Structure and expression of the Xenopus retinoblastoma gene. *Develop Biol* 1992; 153:141-149.
- Diller L, Friend SH. Identification of cancer-prone individuals: p53 and family cancer syndromes. *J. Natl Cancer Inst Monogr* 1992; 12:123-124.
- Frebourg T, Friend SH. Cancer Risks from Germline p53 Mutations. *J Clin Invest* 1992; 90:1637-1641.
- Li FP, Garber JE, Friend SH, Strong LC, Patenaude AF, Juengst ET, Reilly PR, Correa P, Fraumeni JF Jr. Recommendations on predictive testing for germ line p53 mutations among cancer-prone individuals. *J Natl Cancer Inst* 1992; 84:1156-1160.
- Frebourg T, Barbier N, Kassel J, Ng Y, Romero P, Friend SH. A functional screen for germline p53 mutations based on transcriptional activation. *Cancer Research* 1992; 52: 6976-6978.
- Ishioka C, Frebourg T, Yan Y-X, Vidal M, Friend S, Schmidt S, Iggo R. Screening patients for heterozygous p53 mutations using a functional assay in yeast. *Nature Genetics* 1993. In press.

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME	POSITION TITLE
David H. GELFAND	Director, Program in Core Research

EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Brandeis University, Waltham	A.B.	1966	Biology
Univ. of California, San Diego, La Jolla	Ph.D.	1970	Biology
Univ. of California, San Diego, La Jolla	Postdoc.	70-71	Molecular Genetics
Univ. of California, San Francisco, San Francisco	Postdoc	72-76	Molecular Biology

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

1970-1971 UCSD, Postdoctoral Fellow & Research Associate; M. Hayashi  
 1972-1976 UCSF, Research Biochemist; G.M. Tomkins (through 8/76) & W.J. Rutter (through 1/77)  
 1976-1991 Cetus Corp., Director, Recombinant Molecular Research, Senior Scientist, Vice President, Scientific Affairs & Director, Core Technology, PCR Division  
 1991-present Roche Molecular Systems, Director, Program in Core Research

*Honors and Awards:*

New York State S.E. Regional Science Fair, First Prize Winner, Senior Division and Grand Prize Winner, 1962  
 New York State Science Fair Finalist Sixth Prize, 1962  
 Awarded New York State four-year full tuition scholarship (award not accepted), 1962  
 IPO "Distinguished Inventor Award", Senate Office Building, 1990

*Representative Publications:*

Rousseau, G.G., Higgins, S.J., Baxter, J.D., Gelfand, D.H., and Tomkins, G.M. (1975). Binding of glucocorticoid receptors to DNA. *J. Biol. Chem.*, **250**:6015-6021.  
 Polisky, B., Bishop, R.J., and Gelfand, D.H. (1976). A plasmid cloning vehicle allowing regulated expression of eukaryotic DNA in bacteria. *Proc. Natl. Acad. Sci. USA*, **73**:3900-3904.  
 Gelfand, D.H., and Steinberg, R.A. (1977). Mutants of *Escherichia coli* deficient in the aspartate and aromatic amino acid aminotransferases. *J. Bacteriol.*, **130**:429-440.  
 Gelfand, D.H., Shepard, H.M., O'Farrell, P.H., and Polisky, B. (1978). Isolation and characterization of a ColEI-derived plasmid copy-number mutant. *Proc. Natl. Acad. Sci. USA*, **75**:5869-5873.  
 Shepard, H.M., Gelfand, D.H., and Polisky, B. (1979). Analysis of a recessive plasmid copy number mutant: Evidence for negative control of ColEI replication. *Cell*, **18**:267-275.

- Innis, M.A., Holland, M.J., McCabe, P.C., Cole, G.E., Wittman, V.P., Tal, R., Watt, K.W.K., Gelfand, D.H., Holland, J.P., and Meade, J.H. (1985). Expression, glycosylation, and secretion of an aspergillus glucoamylase by *Saccharomyces cerevisiae*. *Science*, **228**:21-26.
- Erlich, H.A., Gelfand, D.H., and Saiki, R.K. (1988). Specific DNA Amplification. *Nature*, **331**:461-462.
- Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. (1988). Primer-Directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase. *Science*, **239**:487-491.
- Innis, M.A., Myambo, K.B., Gelfand, D.H., and Brow, M.A.D. (1988). DNA Sequencing with *Thermus aquaticus* DNA Polymerase, and Direct Sequencing of PCR-amplified DNA. *Proc. Natl. Acad. Sci. USA*, **85**:9436-9440.
- Lawyer, F.C., Stoffel, S., Saiki, R.K., Myambo, K., Drummond, R., and Gelfand, D.H. (1989). Isolation, Characterization, and Expression in *Escherichia coli* of the DNA Polymerase Gene from *Thermus aquaticus*. *J. Biol. Chem.*, **264**:6427-6437.
- Gelfand, D.H. (1989). *Taq* DNA Polymerase. In: *PCR Technology: Principles and Applications for DNA Amplification*. (Erlich, H.A., ed.), Stockton Press, New York, N.Y., pp. 17-22.
- Innis, M.A., Gelfand, D.H., Sninsky, J.J., and White, T.J., eds. (1990). *PCR Protocols: A Guide to Methods and Applications*, Academic Press, San Diego, CA.
- Innis, M.A. and Gelfand, D.H. (1990). Optimization of PCRs. In: *PCR Protocols: A Guide to Methods and Applications*. *ibid.* pp. 3-12.
- Gelfand, D.H. and White, T.J. (1990). Thermostable DNA Polymerases. In: *PCR Protocols: A Guide to Methods and Applications*. *ibid.* pp. 129-141.
- Wong, H.C., Fear, A.L., Calhoon, R.D., Eichinger, G.H., Mayer, R., Amikam, D., Benziman, M., Gelfand, D.H., Meade, J.H., Emerick, A.W., Bruner, R., Ben-Bassat, A., and Tal, R. (1990). Genetic Organization of the Cellulose Synthase Operon in *Acetobacter xylinum*. *Proc. Natl. Acad. Sci. USA*, **87**:8130-8134.
- Erlich, H.A., Gelfand, D.H., and Sninsky, J.J. (1991). Recent Advances in the Polymerase Chain Reaction. *Science* **252**:1643-1651.
- Myers, T.W. and Gelfand, D.H. (1991). Reverse Transcription and DNA Amplification by a *Thermus thermophilus* DNA Polymerase. *Biochemistry* **30**:7661-7666.
- Holland, P.M., Abramson, R.D., Watson, R., and Gelfand, D.H. (1991). Detection of Specific Polymerase Chain Reaction Product by Utilizing the 5'  $\rightarrow$  3' Exonuclease Activity of *Thermus aquaticus* DNA Polymerase. *Proc. Natl. Acad. Sci. USA* **88**:7276-7280.
- Barany, F. and Gelfand, D.H. (1991). Cloning, Overexpression and Nucleotide Sequence of a Thermostable DNA Ligase-Encoding Gene. *Gene* **109**:1-11.
- Lawyer, F.C., Stoffel, S., Saiki, R.K., Chang, S., Landre, P.A., Abramson, R.D., and Gelfand, D.H. (1993). High-level Expression, Purification, and Enzymatic Characterization of Full-length *Thermus aquaticus* DNA Polymerase and a Truncated Form Deficient in 5' to 3' Exonuclease Activity. *PCR Methods and Applications* **2**:275-287.

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME Aaron GILES		POSITION TITLE Programmer/Analyst II	
EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
University of Chicago	B.S.	1992	Physics

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

Office of Academic Computing, Cornell University Medical College  
Implemented an interface for acquiring and documenting medical images from external video sources.  
Co-developed the Cornell University IP-based videoconferencing system, CU-SeeMe.  
July, 1993 to present

*Junior Programmer/Physicist*

High Energy Physics Department (OPAL group), The University of Chicago and CERN  
Designed and implemented firmware for a set of new, high-speed VME-based readout electronics at CERN.  
Developed a test suite for running low-level diagnostics on the custom electronics in an OS/9 environment.  
Assisted in the debugging and full testing of the hardware modules.  
June, 1992 to June, 1993

*Research and Computing Assistant*

High Energy Physics Department (OPAL group), The University of Chicago  
Analyzed data from the OPAL particle detector at CERN for rare interactions of fundamental particles.  
Published an internal CERN Technical Note describing the results of the above analysis.  
January, 1991 to June, 1992

*Local Computer Chairman*

Woodward Court and University Computing Organizations, The University of Chicago  
Provided approximately 200 Macintosh users with computer-related advice and assistance.  
Volunteered two hours per week to the on-campus support hotline, servicing over 5,000 users.  
Assisted the department in disassembling and examining the Michaelangelo virus.  
Maintained a public Macintosh cluster and a file/printing server.  
October, 1991 to June, 1992

*Honors and Awards:*

Associate member, Sigma Xi honors society  
Grade Point Average: 3.7/4.0  
Dean's List, 1989-1992  
National Merit Scholar, 1989-1992

## COMPUTER SKILLS

### *Macintosh*

Authored JPEGView, a free QuickTime-based image viewer written in C and 68020 assembly. Programming experience includes: QuickTime, image compression, floating palettes, AppleScript, the AppleEvent Object Model, direct-to-screen drawing, and hand-optimized 68020 assembly.

### *MS-DOS*

Authored QuickASM, a proprietary self-compiling 8086 assembler written in 8086 assembly. Programming experience includes: TSR utilities, interrupt patching, hand-coded 8086 assembly, data compression, printer drivers, CGA/VGA graphics, and serial port communications.

### *General/Other Platforms*

Strong background in speed optimization using hand-coded assembly (both Motorola 680x0 and Intel 80x86).

Extensive coding experience in both C, C++ and FORTRAN on Unix and VAX/VMS workstations. Experienced in the use of the Internet, database management software, assistance, and user support.

## BIOGRAPHICAL SKETCH

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME Lawrence I. GROSSMAN		POSITION TITLE Professor	
EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
City College of New York, New York	B.S.	1961	Biochemistry
Albert Einstein College of Medicine, New York	Ph.D.	1970	Genetics
California Institute of Technology, California	PostDoc.	70-74	Molecular Biology

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

1966-70 Graduate student, Albert Einstein College of Medicine, Bronx, NY 10463. Advisor: Prof. Julius Marmur.

1970-74 Research Fellow, Division of Biology, California Institute of Technology, Pasadena 91125. Advisor: Prof. Jerome Vinograd.

1974-78 Assistant Professor of Biochemistry, Wayne State University School of Medicine, Detroit, MI.

1978-85 Assistant Professor of Biological Sciences, The University of Michigan, Ann Arbor 48109.

1985-86 Visiting Scientist, NIMH, National Institutes of Health, Bethesda, MD 20205.

1985-86 Senior Editor, *Science* Magazine, 1333 H Street, N.W., Washington, DC 20005.

1986-present Positions in the Department of Molecular Biology and Genetics, Wayne State University School of Medicine (**Boldface=current**). Associate Professor, 1986-91; **Professor, 1991-present**; Acting Chairman, April-Nov., 1992; **Associate Chairman, Nov. 1992-present.**

*Honors and Awards:*

Fabrege Award in Chemistry, City College of New York, 1961; Research Award, City College Chemistry Alumni Association, 1961; Samuel Rubin Prize, City College of New York, 1961; Pre-doctoral Fellow of the National Institutes of Health, 1968-69; Fellow of the Jane Coffin Childs Memorial Fund for Medical Research, 1971-73; Membership, Society of Sigma Xi, 1972-; Membership, American Society for Biochemistry and Molecular Biology, 1978-; *Ad hoc* Member, NIH Study Section (Panel A), 1981; Sigma Xi Lecturer, General Motors Research Laboratories, 1982; Editorial Board, *Electrophoresis*, 1987-92; Contributing Editor, *Science*, 1987-; Editorial Board, *Theoretical and Applied Electrophoresis*, 1988-9; Associate Editor, *Theoretical and Applied Electrophoresis*, 1989-; Consulting Editor, *McGraw-Hill Encyclopedia of Science and Technology*, 1988-; National Science Foundation Expert Review Panel, 1988; Treasurer, American Electrophoresis Soc., 1989-; Dept. of Energy Molecular and Cell Biology Peer Review Panel, 1990; Scientific Advisory Comm. on Molecular Biology and Genetics, American Cancer Soc., 1991; NIH Project Site Visit Panel, 1993.

*Representative Publications:*

R.A. Butow, P.S. Perlman, L.I. Grossman (1985). The unusual *var1* gene of yeast mitochondrial DNA. *Science* **228**, 1496-1501.

D.S. Shumard, L.I. Grossman, M.E.S. Hudspeth (1986). *Achlya* mitochondrial DNA: gene localization and analysis of inverted repeats. *Molec. Gen. Genet.* **202**, 16-23.

- N.J. Bachman, M.I. Lomax, L.I. Grossman (1987). Two bovine genes for cytochrome *c* oxidase subunit IV: a processed pseudogene and an expressed gene. *Gene*, **55**, 219-229.
- M. Zeviani, A. Miranda, J. Herbert, M.I. Lomax, L.I. Grossman, A. Sherbany, S. DiMauro, E.A. Schon (1987). Isolation of a cDNA clone encoding subunit IV of human cytochrome *c* oxidase. *Gene*, **55**, 205-217.
- G.M. Fabrizi, H. Nakase, S. Mita, M.I. Lomax, L.I. Grossman, E.A. Schon (1989). Sequence of a cDNA specifying subunit VIIa of human cytochrome *c* oxidase. *Nucleic Acids Res.* **17**, 7107.
- M.I. Lomax, M.D. Welch, B.T. Darras, U. Francke, L.I. Grossman (1989). Novel use of a chimpanzee pseudogene for chromosomal mapping of human cytochrome *c* oxidase subunit IV. *Gene* **86**, 209-216.
- M.S. Aqua, M.I. Lomax, E.A. Schon, L.I. Grossman (1989). Nucleotide sequence of a cDNA for bovine cytochrome *c* oxidase subunit VIIc. *Nucleic Acids Res.* **17**, 8376.
- L.I. Grossman (1990). Invited editorial: mitochondrial DNA in sickness and in health. *Am. J. Hum. Genet.* **46**, 415-417.
- L.I. Grossman and M. Akamatsu (1990). Sequence of a mouse cDNA for subunit IV of cytochrome *c* oxidase. *Nucleic Acids Res.* **18**, 6454.
- M.S. Aqua, N.J. Bachman, M.I. Lomax, L.I. Grossman (1991). Characterization and expression of a cDNA for subunit VIIc of bovine cytochrome oxidase. *Gene* **104**, 211-217.
- R.S. Seelan and L.I. Grossman (1991). Cytochrome *c* oxidase subunit VIIa isoforms: characterization and expression of bovine cDNAs. *J. Biol. Chem.* **266**, 19752-19757.
- B.C. White, R.C. Tribhuwan, D.J. Vander Laan, D.J. DeGracia, G.S. Krause, L.I. Grossman (1992). Brain mitochondrial DNA is not damaged by prolonged cardiac arrest or reperfusion. *J. Neurochem.* **58**, 1716-1722.
- M.I. Lomax, D. Hewett-Emmett, T. Yang, L.I. Grossman (1992). Rapid evolution of the human gene for cytochrome *c* oxidase subunit IV. *Proc. Nat. Acad. Sci. USA* **89**, 5266-5270.
- R.S. Seelan and L.I. Grossman (1992). Structure and organization of the heart isoform gene for bovine cytochrome *c* oxidase subunit VIIa. *Biochemistry* **31**, 4696-4704.
- D.J. DeGracia, B.J. O'Neil, G.S. Krause, J.M. Skjaerlund, B.C. White, L.I. Grossman (1993). Studies of protein synthesis in the brain cortex during global ischemia and reperfusion. *Resuscitation* **25**, 161-170.
- B.C. White, G.S. Krause, L.I. Grossman (1993). Membrane damage and repair in brain injury by ischemia and reperfusion. *Neurology*, in press.
- D.J. DeGracia, B.J. O'Neil, B.C. White, B.R. Tiffany, G.S. Krause, L.I. Grossman, G. Grunberger (1993). Insulin induces tyrosine phosphorylation of a 90-kDa protein during post-ischemic brain reperfusion. *Exp. Neurology* **124**, In press.
- A. Ao, R.P. Erickson, N.H. Rosenthal, L.I. Grossman (1993). Antisense inhibition of nuclear-encoded cytochrome *c* oxidase subunits IV and VIIc in the pre-implantation embryo. *Dev. Genet.* **14**, In press.
- R.S. Seelan and L.I. Grossman (1993). Structural organization and evolution of the liver isoform gene for bovine cytochrome *c* oxidase subunit VIIa. *Genomics*, In press.

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME Neil R. HACKETT		POSITION TITLE Assistant Professor	
EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
University of Edinburgh, Scotland	B.Sc.	1978	Biochemistry
University of British Columbia, Canada	Ph.D.	1982	Biochemistry
MIT, Cambridge, MA	Postdoc.	82-86	Mol. Biol.

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

- 1/90 - Present Manager of Molecular Biology Computing Facility, CUMC.
- 7/89 - Present Assistant Professor, Department of Microbiology,  
Cornell University Medical College, New York NY.
- 9/86 - 6/89 Assistant Professor, Department of Molecular Biology,  
Vanderbilt University, Nashville TN.
- 10/82 - 8/86 Post-doctoral Fellow, Department of Chemistry,  
Massachusetts Institute of Technology, Cambridge MA.

*Honors and Awards:*

- 1990 - 1993 Cornell Scholarship in Biomedical Sciences
- 1989 - 1993 NIH First Award
- 1982 - 1984 Damon Runyon-Walter Winchell Cancer Fund Fellowship
- 1978 - 1982 Commonwealth Scholarship
- 1975 - 1978 Science Faculty Bursary, University of Edinburgh

*Representative Publications:*

- Bobovnikova, Y., DasSarma, S., Ng, W-L. and Hackett, N.R. (1993). Top-down restriction mapping the genome of Halobacterium halobium strain NRC-1. *System. Appl. Microbiol.* Accepted for publication.
- Hackett, N.R. (1993). Preparation of intact DNA from Halobacterium halobium and its digestion by restriction enzymes. in "*Protocols for Archaeal Research*" (S. DasSarma Ed.) Cold Spring Harbor Press.
- Hackett, N.R. and Ken, R. (1993). Electrophoretic mobility shift assays with crude extracts of halobacterium halobium. In "*Protocols for Archaeal Research*" (S. DasSarma Ed.) Cold Spring Harbor Press.
- Ken, R. and Hackett N.R. (1991). Halobacterium halobium lysogenic for phage phiH contain a protein resembling coliphage repressors. *J. Bacteriol.* 173:955-960.



- Hackett, N.R., Krebs, M.P., DasSarma, S., Goebel, W., RajBhandary, U.L. and Khorana, H.G. (1990). Nucleotide sequence of a high copy number plasmid from Halobacterium strain GRB. *Nucl. Acids Res.* 18:3408.
- Hall, M.J. and Hackett, N.R. (1989). DNA sequence of a small plasmid of Halobacterium strain GN101. *Nucl. Acids Res.* 17:10501.
- Jones, J.G., Hackett, N.R., Halladay, J.T., Scothorn, D.S., Yang, C.-F., Ng, W.L. and DasSarma (1989). Analysis of insertion mutants reveals two new genes in the plasmid-encoded gas vesicle gene cluster of Halobacterium halobium. *Nucl. Acids Res.* 16:8477-8482.
- Hackett, N.R. and DasSarma, S. (1989). Characterization of the small endogenous plasmid of Halobacterium SB3 and its use in transformation of H. halobium. *Can. J. Microbiol.* 35:86-91.
- Braiman, M.S., Mogi, T., Stern, L.J., Hackett, N.R., Chao, B.H., Khorana, H.G. and Rothschild, K.J. (1988). Vibrational spectroscopy of bacteriorhodopsin mutants. I. Tyrosine 185 deprotonates and reprotonates during the photocycle. *Proteins* 3:219-229.
- Mogi, T., Stern, L.J., Hackett, N.R. and Khorana, H.G. (1987). Bacterio-rhodopsin mutants containing single tyrosine to phenylalanine substitutions are all active in proton pumping. *Proc. Natl. Acad. Sci. U.S.A.* 84:5595-5599.
- Hackett, N.R., Stern, L.J., Chao, B.H., Kronis, K.A., and Khorana, H.G. (1987). Structure-function studies on bacteriorhodopsin. V. Effect of amino acid substitutions in the putative helix F. *J. Biol. Chem.* 262:9277-9284.
- Dunn, R.J., Hackett, N.R., McCoy, J.M., Chao, B.H., Kimura, K. and Khorana, H.G. (1987). Structure-function studies on bacteriorhodopsin. I. Expression of the bacterio-opsin gene in E. coli. *J. Biol. Chem.* 262:9246-9254.
- Lo, K.-M., Jones, S.J., Hackett, N.R. and Khorana, H.G. (1984). Specific amino acid substitutions in bacteriorhodopsin: Replacement of a restriction fragment in the structural gene by synthetic DNA containing altered codons. *Proc. Natl. Acad. Sci. U.S.A.* 81:2285-2289.

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME <b>Robert P. HAMMER</b>		POSITION TITLE <b>Assistant Professor</b>	
EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
University of Illinois, Urbana, IL	B.S.	1985	Chemistry
University of Minnesota, Minneapolis, MN	Ph.D.	1990	Organic Chemistry
Swiss Federal Institute of Technology (ETH), Zurich, Switz.	postdoc	90-92	Bioorganic Chemistry

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

**Professional Experience:**

January - May 1983, August - December 1983, May - August 1984, **Cooperative Education Chemist**, Nalco Chemical Company, Naperville, IL.

September 1990 - June 1992, **Postdoctoral Fellow**, Swiss Federal Institute of Technology (ETH) (Professor Dr. Albert Eschenmoser), Zurich, Switzerland.

August 1992 - Present, **Assistant Professor**, Department of Chemistry, Louisiana State University, Baton Rouge, LA.

Dr. Hammer has expertise in the synthesis, isolation, and biophysical characterization of mononucleoside and oligonucleotide analogs. His work with Professor Eschenmoser in Zurich, Switzerland involved *de novo* synthesis of 2 classes of hexose-based, protected nucleosides and incorporation of these derivatives into oligonucleotides. These hexose-based nucleic acids were characterized by melting temperature experiments (temperature-dependent UV) and circular dichroism spectroscopy. This work is currently being prepared for publication in *Helvetica Chimica Acta* and should appear in the beginning of 1994.

**Honors and Awards:**

College of Basic Sciences (LSU) Student Council Teacher Award, 1993  
 Amoco Foundation Fellow, 1989-1990  
 Lee Irvin Smith Award for High Ability and Leadership in Organic Chemistry, 1989  
 University of Minnesota Graduate School Dissertation Fellow, 1987-1988  
 University of Minnesota Graduate School Fellow, 1985-1987  
 Phi Beta Kappa  
 Phi Lambda Upsilon

**Representative Publications:**

Scott E. Denmark, Robert P. Hammer, Eric J. Weber and Karl L. Habermas, "Diphenylmethylsilyl Ether (DPMS): A Protecting Group for Alcohols," *J. Org. Chem.* 52, 165-168 (1987).

Fernando Albericio, Nancy Kneib-Cordonier, Lajos Gera, Robert P. Hammer, Derek Hudson and George Barany, "Solid-Phase Synthesis of Peptide Amides Under Mild Conditions," In *Peptides - Chemistry and Biology: Proceedings of the Tenth American Peptide Symposium* (G.R. Marshall, ed.), Escom Science Publishers, Leiden, The Netherlands, 1988, pp. 159-161.

- C. García-Echeverría, R.P. Hammer, M.A. Molins, F. Albericio, M. Pons, G. Barany and E. Giralt, "Cyclization of Disulfide-Containing Peptides in Solid-Phase Synthesis," In *Peptides – Chemistry and Biology: Proceedings of the Eleventh American Peptide Symposium* (J. Rivier & G.R. Marshall, eds.), Escom Science Publishers, Leiden, The Netherlands, 1990, pp. 996-998.
- R.P. Hammer, F. Albericio, L. Gera and G. Barany, "Practical Approach to Solid-Phase Synthesis of C-Terminal Peptide Amides Under Mild Conditions Based on a Photolysable Anchoring Linkage," *Int. J. Peptide Protein Res.* 36, 31-45 (1990).
- F. Albericio, R.P. Hammer, C. García-Echeverría, M.A. Molins, J.L. Chang, M.C. Munson, M. Pons, E. Giralt and G. Barany, "Cyclization of Disulfide-Containing Peptides in Solid-Phase Synthesis," *Int. J. Peptide Protein Res.* 37, 402-413 (1991). C.R. Johnson, S. Biancalana, R.P. Hammer, P.B. Wright, and D. Hudson, "New Active Esters and Coupling Reagents Based on Pyrazolinones," In *Peptides – Chemistry and Biology: Proceedings of the Twelfth American Peptide Symposium* (J. Smith & J. Rivier, eds.), Escom Science Publishers, Leiden, The Netherlands, 1991, pp. 585-586.
- D. Hudson, C.R. Johnson, G. Barany, S. Biancalana, B.J. Calnan, A.D. Frankel, W.B. Cohn, T. Hayes, C. Dahl, M.A. Markus, M.A. Weiss, R.P. Hammer, H.-t. Hsu, R. Jordan, K.K. Kamo, M.H. Lytle, L. Toll, D.S. Tsou, and P.B. Wright, "Tactics and Strategies in Solid-Phase Peptide Synthesis: New Directions, Methods and Applications," In *Innovations and Perspectives in Solid Phase Synthesis and Related Technologies: Peptides, Polypeptides, and Oligonucleotides 1992* (R. Epton, ed.), Intercept, Andover, England, 1992, pp. 135-152.

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

<b>NAME</b> Eric P. HOFFMAN	<b>POSITION TITLE</b> Assistant Professor		
<b>EDUCATION</b> (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
<b>INSTITUTION AND LOCATION</b>	<b>DEGREE</b>	<b>YEAR CONFERRED</b>	<b>FIELD OF STUDY</b>
Gettysburg College, Gettysburg, PA	BA	1982	Biology
Gettysburg College, Gettysburg, PA	BA	1982	Music
Johns Hopkins University, Baltimore, MD	Ph.D.	1987	Biology
Harvard Medical School, Boston, MA	Fellow	1989	Pediatrics

**RESEARCH AND/OR PROFESSIONAL EXPERIENCE:** Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

**Research Fellowships:**

- 1986-90 Research Fellow in Medicine, Children's Hospital, Boston
- 1986-88 Research Fellow in Pediatrics, Harvard University, Boston
- 1988-90 Research Associate, Howard Hughes Medical Institute, Boston

**Faculty Appointments:**

- 1988-89 Instructor in Pediatrics, Harvard University
- 1989-90 Assistant Professor in Pediatrics, Harvard University
- 1990- Assistant Professor, University of Pittsburgh  
Department of Molecular Genetics & Biochemistry, School Medicine  
Department of Human Genetics, School of Public Health,  
Department of Pediatrics, School of Medicine, Children's Hospital

**Awards and Honors:**

- 1982 Darrah Award in Biology, Gettysburg College
- 1985 Genetics Society of America, Graduate Student Travel Award
- 1986-1988 Zimmerman Postdoctoral Fellowship, Muscular Dystrophy Association
- 1990- Scientific Advisory Committee, Telethon Italian Muscular Dystrophy
- 1991 The Young Alumni Achievement Award, Gettysburg College
- 1991 "G. Conte" Award, University of Naples, Italy

**Selected Publications since 1987: (from 96)**

- Hoffman, EP, Monaco, AP, Feener, CC, Kunkel, LM. Conservation of the Duchenne muscular dystrophy gene in mice and humans. *Science* 1987; 238: 347-350.
- Koenig, M, Hoffman, EP, Bertelson, CJ, Monaco, AP, Feener, C, and Kunkel, LM. Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. *Cell* 1987; 50: 509-517.
- Hoffman, EP, Brown, RH, and Kunkel, LM. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. *Cell* 1987; 51: 919-928.
- Hoffman, EP, Knudson, CM, Campbell, KP, and Kunkel, LM. Subcellular fractionation of dystrophin to the triads of skeletal muscle. *Nature* 1987; 330: 754-758.
- Hoffman, EP, et al. Dystrophin characterization in muscle biopsies from Duchenne and Becker muscular dystrophy patients. *New Eng J Med* 1988; 318: 1363-1368.

- Hoffman, EP, Hudecki, M, Rosenberg, P, Pollina, C, and Kunkel, LM. Cell and fiber -type distribution of dystrophin. *Neuron* 1988; 1: 411-420.
- Hoffman, EP, Kunkel, LM, Angelini, C, Clarke, A, Johnson, M, and Harris, JB. Improved diagnosis of Becker muscular dystrophy by dystrophin testing. *Neurology* 1989; 39: 1011-1017.
- Hoffman, EP, and Kunkel, LM. Dystrophin abnormalities in Duchenne/Becker muscular dystrophy. *Neuron* 1989; 2: 1019-1029.
- Hoffman, EP, Morgan, JE, Watkins, SC, and Partridge, TA. Somatic reversion/suppression of the mouse *mdx* phenotype *in vivo*. *J Neurol Sci* 1990; 99: 9-25.
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- Hoffman, EP, Garcia, CA, Chamberlain, JS, Angelini, C, Lupski, JR, and Fenwick, R. Is the carboxyl-terminus required for membrane association? A novel, severe case of Duchenne muscular dystrophy. *Ann Neurol* 1991; 30: 605-610.
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- Koch, MC, Ricker, K, Otto, M, Grimm, T, Bender, K, Zoll, B, Lehmann-Horn, F, Rudel, R, Harper, PS, and Hoffman, EP. Paramyotonia congenita and hyperkalemic periodic paralysis are allelic disorders on chromosome 17. *Hum Genet* 1991; 88: 71-74.
- Rojas, CV, Wang, JZ, Schwartz, LS, Hoffman, EP, Powell, BR, and Brown, RH. A methionine to valine mutation in the skeletal muscle sodium channel alpha-subunit in human hyperkalemic periodic paralysis. *Nature* 1991; 354: 387-389.
- Wang, JZ, Rojas, CV, Zhou, J, Schwartz, LS, Nicholas, N, and Hoffman, EP. Sequence and genomic structure of the human adult skeletal muscle sodium channel alpha subunit gene on 17q. *Biochem Biophys Res Com* 1992; 182: 794-801.
- Rudolph, JA, Spier, SJ, Byrns, G, and Hoffman, EP. Linkage of hyperkalemic periodic paralysis in Quarter Horses to the horse adult skeletal muscle sodium channel gene. *Animal Genet* 1992; 23: 241-250.
- Schwartz, LS, Tarleton, J, Popovich, B, Seltzer, W, and Hoffman, EP. PCR-based linkage analysis and carrier detection for Duchenne and Becker muscular dystrophies using an automated sequencer. *Am J Hum Genet* 1992; 51: 721-729.
- Rudolph, JA, Spier, SJ, Byrns, G, Rojas, CV, Bernoco, D, and Hoffman, EP. A sodium channel Phe->Leu mutation in hyperkalemic periodic paralysis in Quarter Horses: a common defect disseminated by selective breeding of a popular sire. *Nature Genet* 1992; 2: 144-147.
- Feero, WT, Wang, J, Barany, F, Zhou, J, Todorovic, SM, Conwit, R, Galloway, G, Hausmanowa-Petrusewicz, I, Fidzianska, A, Arahata, K, Wessel, HB, Sillen, A, Marks, HG, Hartlage, P, Hayakawa, H, and Hoffman, EP. Hyperkalemic Periodic Paralysis: Rapid Molecular Diagnosis and Relationship of Genotype to Phenotype in 12 Families. *Neurology* 1993; 43: 668-673.
- Wang, J, Zhou, J, Todorovic, SM, Feero, WG, Barany, F, Conwit, R, Galloway, G, Fidzianska, A, Hausmanowa-Petrusewicz, I, Arahata, K, Wessel, HB, Wadelius, C, Marks, HG, Hartladge, P, Lehmann-Horn, F, and Hoffman, EP. Molecular genetic and genetic correlations in sodium channelopathies: Lack of founder effect and evidence for a second gene. *Am J Hum Genet* 1993; 52: 1074-1084.
- Hoop, RC, Russo, LS, Riconda, D, Schwartz, LS, and Hoffman, EP. Restoration of half the normal dystrophin sequence in a double-deletion Duchenne muscular dystrophy family. *Am J Med Genet*, *in press*.
- Gorospe, JRM, Tharp, MD, Hinckley, J, Kornegay, JN, and Hoffman, EP. A role for mast cells in the progression of Duchenne muscular dystrophy? Correlations in dystrophin-deficient humans, dogs, and mice. *J Neurol Sci*, *in press*.

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME Timothy C. KENNEDY	POSITION TITLE Associate Clinical Professor, Medical Director
----------------------------	--

EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Yale University	B.A.	1965	English Lit.
University of Arizona	M.S.	1967	Genetics
Columbia University	M.D.	1971	Medicine

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

- 1972 - 74 Intern/Resident, Presbyterian Medical Center
- 1974 - 76 Pulmonary Fellow, University of Arizona
- 1967 Teaching Assistant, University of Arizona, Department of Genetics
- 1975 - 76 Instructor, Department of Medicine, Pulmonary Division, University of Arizona Health Sciences Center
- 1976 - 79 Clinical Instructor, Pulmonary Division, Department of Medicine, University of Colorado Health Sciences Center, Denver, CO
- 1979 - 86 Assistant Clinical Professor, Pulmonary Division, Department of Medicine, University of Colorado Health Sciences Center, Denver, CO
- 1986 - Associate Clinical Professor, Pulmonary Division, Department of Medicine, University of Colorado Health Sciences Center, Denver, CO

*Honors and Awards:*

- Outstanding Teaching Attendant, Internal Medicine, Presbyterian Denver Hospital, 1978.
- Gold Medal, Medley Relay (Yale University), National A.A.U. Championships.
- All-American Collegiate Swimming Team.
- 1972 Diplomat, National Board of Medical Examiners, No. 116026
- 1974 Diplomat, American Board of Internal Medicine, No. 47244
- 1976 Diplomat, Pulmonary Diseases, ABIM, No. 47244
- 1987 Diplomat, Critical Care Medicine, ABIM, No. 47244

*Professional Societies:*

- Denver Medical Society
- Colorado Medical Society, Delegate from DMS to CMS (1978-1981 and 1983-1988)
- American Medical Association
- Presbyterian Medical Center, Housestaff Association (President, 1973)
- Arizona Thoracic Society (1974-1976)
- Arizona Lung Association (1975-1976)
- American Thoracic Society
- Colorado Trudeau Society
- American Lung Association of Colorado, Board of Directors (1977-1978)
- American College of Physicians, Associate (1973-1975)
- American College of Chest Physicians, Fellow (1977-1989)
- Colorado Pulmonary Physicians, Vice President/Treasurer (1977-1989)
- National Association of Medical Directors of Respiratory Care, Board of Directors (1988-1990)
- Colorado Society of Internal Medicine
- American Society of Internal Medicine

*Civic and Professional Committees:*

Presbyterian/St. Lukes Center for Health Science Education; Founding Chairman of the Board (1988-present)  
Lung Cancer Institute of Colorado; Founding Chairman of the Board (1989-present)  
Denver Lung Transplant Group (1988-present)  
Colorado Medical Society; Committee on Socioeconomic Affairs (1986-present)  
Colorado Medical Society; Environmental Health Committee (1986-present)  
Columbine Medical Group; Board of Directors (1990-present)  
Denver Medical Practice Association; Board of Directors (1985-1989)  
Presbyterian/St. Lukes Medical Center; Corporate Steering Committee - Internal Medicine (1981-present)

*Bibliography:*

- Kennedy, T.C., and Broughton, J.O. (1977) Exercise-aggravated hypoxemia and orthodeoxia in cirrhosis. *Chest* 72: 3.
- Knudson, R.J., Clark, D., Kennedy, T.C., Knudson, D. (1977) Effect of aging alone on mechanical properties of the normal adult human lung. *J. Appl. Physiol.* 43: 1054-1062.
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- Broughton, J.O., and Kennedy, T.C. (1984) Interpretations of blood gases by computer. *Chest* 85: 148-149.
- Lebowitz, M.D., Burrows, B., Traver, G.A., McDonagh, D.J., Dodge, R.R., Barbee, R.A., Glover, J., Kennedy, T.C., Clark, D., Resar, R. (1985) Methodological considerations of epidemiological diagnoses in respiratory diseases. *Eur. J. Epidemiol.* 1: 188-192.
- Kennedy, T.C., and Broughton, J.O. (1986) Computerization of arterial blood-gas reports and interpretation. *Internal. Med. Specialist* 7: 156-160.
- Hegstron, T., Emmons, L.L., Hoddes, E., Kennedy, T.C., Christopher, K., Collins, T., Spofford, B. (1988) Obstructive sleep apnea syndrome: Preoperative radiologic evaluation. *Am. J. Radiol.* 150: 67-69.
- Kennedy, T.C. (1989) Current therapy of respiratory disease-3. In High Altitude Diseases, Cherniack, R.M., ed.
- Kennedy, T.C. (1991) Current therapy of internal medicine-3. In High Altitude Diseases, Kassirer, ed.
- Schaiberger, P.H., Kennedy, T.C., Miller, F.C., Gal, J., Petty, T.L. (1993) Pulmonary hypertension associated with long term inhalation of "CRANK" methamphetamine. *Chest* 104: 614-616.
- Franklin, W.A., Kennedy, T.C., Miller, Y., Meyer, A., Folkvoord, J., Garza-Williams, S., Parks, T., Bunn, P.A.: Expression of epidermal growth factor receptor, neural cell adhesion molecule, Ki-67 and transferrin receptor by dysplastic bronchial epithelium. *Int. J. Cancer* (In Press).

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME	POSITION TITLE
Olen KEW	Chief, Molecular Virology Section Div. Viral Dis./Nat. Ctr. Infect. Dis./CDC

**EDUCATION** (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
University of California, Irvine	B.S.	1968	Biological Sciences
University of Washington, Seattle	M.S.	1972	Microbiology
University of Washington, Seattle	Ph.D.	1974	Microbiology

**RESEARCH AND/OR PROFESSIONAL EXPERIENCE:** Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

**Professional Experience:**

1974-1979 University of Wisconsin, Biophysics Laboratory, Postdoctoral Fellow: R. Rueckert.  
1979-1984 Centers for Disease Control & Prevention, Senior Staff Fellow, Senior Research Chemist  
1984-present Centers for Disease Control & Prevention, Chief, Molecular Virology Section

**Honors and Awards:**

President's Fellow for Undergraduate Research, University of California, 1967-68.  
National Institutes of Health Postdoctoral Research Fellow, (NIAID), 1975-77.  
Charles C. Shepard Science Award, Centers for Disease Control, 1988.  
United States Public Health Service Special Recognition Award, 1989.  
United States Department of Health and Human Services Distinguished Service Award, 1991.

**Representative Publications:**

Rico-Hesse, R., Pallansch, M.A., Nottay, B.K., and Kew, O.M. (1987). Geographic distribution of wild poliovirus type 1 genotypes. *Virology* **160**, 311-322.

Slater, P.E., Orenstein, W.A., Morag, A., Avni, A., Handscher, R., Green, M.S., Costin, C., Yarrow, A., Rishpon, S., Havkin, O., Ben-Zvi, T., Kew, O.M., Rey, M., Epstein, I., Swartz, T.A., and Melnick, J.L. (1990). Poliomyelitis outbreak in Israel in 1988: a report with two commentaries. *Lancet* **335**, 1192-1198.

Kew, O.M., Nottay, B.K., Rico-Hesse, R.R., and Pallansch, M.A. (1990). Molecular epidemiology of wild poliovirus transmission. In: *"Applied Virology Research"* (E. Kurstak, R.G. Marusyk, F.A. Murphy, and M.H.V. Van Regenmortel, eds.), Vol. 2, Plenum Press, New York, pp. 199-221.

Pöyry, T., Kinnunen, L., Kapsenberg, J., Kew, O., and Hovi, T. (1990). Type 3 poliovirus/Finland/1984 is genetically related to common Mediterranean strains. *J. Gen. Virol.* **71**, 2535-2541.

da Silva, E.E., Pallansch, M.A., Holloway, B.P., Cuoto Oliveira, M.J., Schatzmayr, H.G., and Kew, O.M. (1990). Oligonucleotide probes for the specific detection of the wild poliovirus types 1 and 3 endemic to Brazil. *Intervirology* **32**, 149-159.

Yang, C.-F., De, L., Holloway, B.P., Pallansch, M.A., and Kew, O.M. (1991). Detection and identification of vaccine-related polioviruses by the polymerase chain reaction. *Virus Research* **20**, 159-179.



- Muzychenko, A.R., Lipskaya, G.Y., Maslova, S.V., Svitkin, Y.V., Pilipenko, E.V., Nottay, B.K., Kew, O.M., and Agol, V.I. (1991). Coupled mutations in the 5'-untranslated region of the Sabin poliovirus strains during *in vivo* passage: structural and functional implications. *Virus Research* **21**, 111-122.
- Strebel, P.M., Sutter, R.W., Cochi, S.L., Biellik, R.J., Brink, E.W., Kew, O.M., Pallansch, M.A., Orenstein, W.A., and Hinman, A.R. (1992). Epidemiology of poliomyelitis in the United States one decade after the last reported case of indigenous wild virus-associated disease. *Clinical Infectious Diseases* **14**, 568-579.
- Yang, C.-F., De, L., Yang, S.-J., Gómez, J.R., Cruz, J.R., Holloway, B.P., Pallansch, M.A., and Kew, O.M. (1992). Genotype-specific *in vitro* amplification of sequences of the wild type 3 polioviruses from Mexico and Guatemala. *Virus Research*, **24**, 277-296.
- Kew, O.M., De, L., Yang, C.-F., Nottay, B.K., da Silva, E., and Pallansch, M.A. (1993). The role of virologic surveillance in the global initiative to eradicate poliomyelitis. In: "*Control of Virus Diseases*", 2nd ed. (E. Kurstak, ed.), pp. 215-246. Marcel Dekker, New York.
- Tambini, G., Andrus, J.K., Marques, E., Boshell, J., Pallansch, M., de Quadros, C.A., and Kew, O. (1993). Direct detection of wild poliovirus transmission by stool surveys of healthy children and analysis of community wastewater. *J. Infect. Dis.* In press.
- Zheng, D.-P., Zhang, L.-B., Fang, Z.-Y., Yang, C.-F., Mulders, M., Pallansch, M.A., and Kew, O.M. (1993). Distribution of wild type 1 poliovirus genotypes in China. *J. Infect. Dis.* In press.

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME John S. KOVACH		POSITION TITLE Professor and Chair, Dept. of Oncology	
EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Princeton University, Princeton, NJ.	B.A.	1958	Biology
Columbia University, New York City.	M.D.	1962	Medicine
Presbyterian Hospital, New York City.		62-65	Med Int & Res.
College of Physicians and Surgeons, NYC.		65-66	USPHS Fellowship

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

1968-1972 Medical Officer, Laboratory of Chemical Biology, NIAMD, NIH  
 1972-1973 Visiting Resident, Division of Medical Oncology, Mayo Clinic  
 1973-1976 Associate Professor of Medicine, College of Physicians and Surgeons, Columbia University  
 Deputy Director, Clinical Oncology, Cancer Research Center at Columbia University  
 Attending Physician, Columbia-Presbyterian Medical Center, New York, NY  
 Member, Scientific Advisory Committee, Cancer Research Center, Columbia University  
 Member, Institutional Committee on Human Investigation, Columbia University  
 1973-1976 Member, Curriculum Committee, College of Physicians and Surgeons of Columbia University  
 1976-present Consultant, Medical Oncology, Mayo Clinic  
 1976-1981 Associate Professor of Oncology, Mayo Medical School, Mayo Clinic  
 1976-1986 Chair of Developmental Oncology Research, Department of Oncology, Mayo Clinic  
 1981-Present Professor of Oncology, Mayo Clinic  
 1986-Present Chair, Department of Oncology; Director, Mayo Comprehensive Cancer Center  
 1988-1992 Member, Cancer Center Support Review Committee, National Cancer Institute  
 1991-1992 Chair, Cancer Center Support Review Committee, National Cancer Institute  
 1991-Present Member, Education Policy Committee, Mayo Foundation

*Honors and Awards:*

Sigma XI, 1958  
 AOA, 1962  
 Diplomate, Board of Internal Medicine, 1973

*Representative Publications:*

van Haelst-Pisani C, Pisani RJ, and Kovach JS: Cancer immunotherapy: Current status of treatment with Interleukin-2 and lymphokine-activated killer cells. *Mayo Clin Proc* 64:451-465, 1989.  
 Moertel CG and Kovach JS: Perspectives: Cancer research and the health insurance industry. *Blue Cross and Blue Shield Medical Report* 2(4):8-9, 1989.  
 Kovach JS and Beart RW, Jr: Cellular pharmacology of fluorinated pyrimidines in vivo in man. *Invest New Drugs* 7:13-25, 1989.

- Lentz SS, McKean DJ, Kovach JS, and Podratz KC: Phenotypic and functional characteristics of mononuclear cells in ovarian carcinoma tumors. *Gynecol Oncol* 34:136-140, 1989.
- Burnham NL, Elcombe SA, Skorlinski CR, Kosanke JL, and Kovach JS: Computer program for handling investigational oncology drugs. *Am J Hosp Pharm* 46:1821-1824, 1989.
- Vuk-Pavlovic S and Kovach JS: Tumor necrosis factor-receptor in MCF-7 cells. *The FASEB Journal* 3:2633-2640, 1989.
- Lentz SS, Kovach JS, McKean DJ, Wieand HS, and Podratz KC: Effector function of lymphokine-activated killer cells and cytotoxic T lymphocytes in ovarian epithelial carcinoma. *Gynecol Oncol* 38:191-196, 1990.
- Ames MM, Richardson RL, Kovach JS, Moertel CG, and O'Connell MJ: Phase I and clinical pharmacologic evaluation of a parenteral hexamethylmelamine formulation. *Cancer Res* 50:206-210, 1990.
- Maruno M, Kovach JS, Kelly PJ, and Yanagihara T: Transforming growth factor- $\beta$ , epidermal growth factor receptor and proliferating potential in benign and malignant gliomas. *J Neurosurg* 75:97-102, 1991.
- Horst H-A, Scheithauer BW, Kelly PJ, and Kovach JS: Immunohistological detection of transforming growth factor- $\beta$  and characterization of lymphoreticular infiltrates in perivascular regions of human glioblastomas. Submitted for publication in *Cancer* (1991).
- van Haelst-Pisani C, Kovach JS, Kita H, Leiferman KM, Gleich GJ, Silver JE, Kale R, and Abrams JS: Administration of IL-2 results in increased plasma concentrations of IL-5 and eosinophilia in patients with cancer. *Blood* 78(6):1538-1544, 1991.
- Kovach JS: Searching for clues to effective use of biologic response modifiers. Editorial. *J NCI* 83:2-3, 1991.
- Patel SR, Kvols LK, Rubin J, O'Connell MJ, Edmonson JH, Ames MM, and Kovach JS: Phase I-II study of pibenzimol hydrochloride (NSC 322921) in advanced pancreatic carcinoma. *Invest New Drugs* 9:53-57, 1991.
- Kovach JS, McGovern RM, Cassady JA, Swanson SK, Wold LE, Vogelstein B, and Sommer SS: Direct sequencing from "touch preps" of human carcinomas: Analysis of p53 mutations in breast carcinomas. *J NCI* 83:1004-1009, 1991.
- O'Connell MJ, Schaid DJ, Ganju V, Cunningham J, Kovach JS, and Thibodeau SN: Current status of adjuvant chemotherapy for colorectal cancer: Can molecular markers play a role in predicting prognosis? Submitted for publication in *Cancer* (March 1991).
- Cunningham J, Lust JA, Schaid DJ, Bren GD, Carpenter HA, Rizza E, Kovach JS, and Thibodeau SN: Expression of p53 and 17p allelic loss in colorectal carcinoma. *Cancer Res* 52:1974-1980, 1992.
- Sommer SS, Cunningham J, McGovern R, Saitoh S, Schroeder J, Wold LE, and Kovach JS: Pattern of p53 gene mutations in breast cancers of women of the midwestern United States. *J NCI* 84(4):246-252, 1992.
- Cheung TH, Webb MJ, Goellner JR, Kovach JS, and Cunningham JM: Detection of p53 antigen expression in cytologic preparations of ovarian carcinomas. Submitted.
- Kovach JS, Svingen PA, and Schaid DJ: Levamisole potentiation of fluorouracil antiproliferative activity mimicked by orthovanadate, an inhibitor of tyrosine phosphatase. *J NCI* 84:515-519, 1992.

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME Matthew B. LUBIN		POSITION TITLE Director, Medical Genetics	
EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Brandeis University, Walham, MA	B.A.	1979	Biochem. & Biol.
UMDNJ, Newark, NJ	M.D.	1984	Medicine
UMDNJ, Newark, NJ (Internship & Residency)		1987	Internal Medicine
UCLA, Cedars-Sinai Medical Center, CA	Fellow	1990	Medical Genetics

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

**Professional Experience:**

1990- 1990 Clinical Instructor, Division of Medical Genetics,  
UCLA School of Medicine, Los Angeles, CA.  
1990- 1993 Clinical Instructor, Department of Medicine, The New York Hospital-Cornell  
University Medical Center, New York, NY.  
1990- Director of Medical Genetics, Strang Cancer Prevention Center  
New York, NY.  
1993- Assistant Professor, Department of Medicine, The New York Hospital-Cornell  
University Medical Center, New York, NY

**Memberships:**

American Association for the Advancement of Science  
American Medical Association  
American College of Physicians  
American Society for Human Genetics

**Publications:**

Goodman MD, Goodman BK, Lubin MB, Rotter JJ, Schreck, RR. Cytogenetic characterization of renal cell carcinoma in von Hippel-Lindau Syndrome. *Cancer* 65:1150-1154, 1990.  
Lubin MB, Wang SJ, Elashoff J, Rotter JJ, Toyoda H. Precise Gene Dosage Determination by Polymerase Chain Reaction: Theory, Methodology and Statistical Approach. *Molecular and Cellular Probes* 5:307-317, 1991.  
Lubin MB, Gruber H, Lachman RS, Rimoin DL. Skeletal abnormalities in the Turner Syndrome, Rosenfeld, RG and Grumbach, MM eds., Marcel Dekker Inc., New York, 1990. pp. 281-300.  
Lubin MB, Lin HJ, Vadheim CM, Rotter JJ. Genetics of Common Diseases of Adulthood: Implications for Prenatal Counseling and Diagnosis. Clinics in Perinatology. *Clinics in Perinatology* 17:889-910, 1990.

## Abstracts:

- Lubin MB, Fleischer D, Rotter JJ. Achalasia in two Iranian siblings documented by video esophagrams. *Clinical Research*, 37:184A, Jan., 1989. Presented at the annual meeting of the Western Society of Pediatric Research, Carmel, CA, Feb., 7-10, 1989.
- Lubin MB, Toyoda H. Gene dosage by PCR co-amplification. *Journal of Cellular Biochemistry*, Supp.13E:297, 1989. Presented at a UCLA symposium, Apr., 3-7, 1989.
- Lubin MB, Wang SJ, Elashoff J, Rotter JJ, Toyoda H. Analysis of gene dosage by quantitative polymerase chain reaction (PCR) co-amplification: possible application to the investigation of loss or amplification of oncogenes. Presented at the annual meeting of the American Society of Human Genetics, Baltimore, MD, Nov., 11-15, 1989.
- Lubin MB, Wang SJ, Elashoff J, Rotter JJ, Toyoda H. Quantitative polymerase chain reaction co-amplification: Possible application to the investigation of gene loss or amplification in neoplasia. Presented at the meeting of the Western Section American Federation for Clinical Research, Carmel, CA, Feb. 6-9, 1990.
- Lubin MB, Rosenthal G, Halper M, Miller DG. A National High Risk Registry for Women with Family Histories of Breast Cancer. Presented at the 8th International Congress of Human Genetics, Washington, DC, Oct. 6-11, 1991.
- Mansfield ES, Stein J, Portina P, Kronick MN, Gilbert E, Lubin MB. Towards a quantitative PCR test for Down's syndrome and other aneuploidies. Presented at the American Society of Human Genetics, San Francisco, CA, Nov. 9-13, 1992.
- Stein J, Mansfield ES, Kronick MN, Grifo J, Gilbert F, Zhi JX, Lubin MB. Simultaneous multilocus gene dosage determination by quantitative fluorescent PCR. Presented at the American Society of Human Genetics, San Francisco, CA, Nov. 9-13, 1992.
- Raffel LJ, Lubin M, Wang SJ, Garber C, Cane P, Karlan B. Age of onset of ovarian cancer and breast cancer as a function of family history. Presented at the American Society of Human Genetics, San Francisco, CA, Nov 9-13, 1992.
- Girardi SK, Lubin MB. Observations on prostate cancer in a breast cancer registry. *American Journal of Human Genetics*, 51(Suppl): 1085, 1992.
- Girardi SK, Lubin MB. Prostate cancer detection by prostate specific antigen and digital rectal examination in men with family histories of prostate cancer. Presented at the American Society of Human Genetics, New Orleans, LA, Oct 5-9, 1993.
- Rosenthal G, Lubin MB. Genetic testing and hereditary cancer: implications for the future. *American Journal of Human Genetics*, 53(3):1637, 1993.

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME Jianying LUO		POSITION TITLE Postdoctoral Associate	
EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Wuhan University, Wuhan, P. R. China	B.S.	1985	Cell Biology
The City university of New York, New York	Ph.D.	1992	Biochemistry
National Institute of Genetics, Mishima, Japan	Guest Sci.	1992	Biochemistry
Cornell University Medical College, New York	Postdoc.	92-93	Molecular Biology

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

**Professional Experience:**

1992 (Jan.-March) National Institute of Genetics, Guest Scientist, with Dr. A. Ishihama  
 1992-present Cornell University Medical College, Postdoctoral Associate, Dept. of Microbiology, with F. Barany

**Honors and Awards:**

Distinguished student, 1982, 1984, 1985 (Wuhan University)  
 Peopel's Scholarship, 1985 (Wuhan University)  
 University Tuition Scholarship, 1990 (CUNY Graduate School)  
 University Fellowship, 1991 (CUNY Graduate School)  
 Beatrice G. Konheim Graduate Scholarship in the Life Sciences, 1992 (Hunter College of CUNY)

**Publications:**

- Luo, J. and Krakow, J. S. (1992) Characterization and epitope mapping of monoclonal antibodies raised against the beta-prime subunit of the *E. coli* RNA polymerase. *J. Biol. Chem.* 267:18175-18181.
- Luo, J., Fujita, N., Ishihama, A., and Krakow, J. S. (1993) The molecular anatomy of the beta-prime subunit of *E. coli* RNA polymerase. Submitted to *J. Biol. Chem.*
- Wiedmann, M., Luo, J., Barany, F. and Batt, C.A. (1993) Detection of *Listeria monocytogenes* by PCR coupled ligase chain reaction (LCR). *PCR Protocols* (accepted).
- Batt, C. A., Wagner, P., Wiedmann, M., Luo, J., and Gilbert, R. (1994) Detection of Bovine Leukocyte Adhesion Deficiency by Nonisotopic Ligase Chain Reaction. *Animal Genetics*. (accepted).
- Wiedman, M., Wilson, W., Czajka, J., Luo, J., Barany, F., Batt, C. (1994) Ligase chain reaction (LCR)-Overview and applications. (Invited Review) *PCR Methods and Applications*. In press.

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME Gary J. MILLER	POSITION TITLE Associate Professor
------------------------	---------------------------------------

EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
State University of New York, Buffalo, NY	B.A.	1972	Biology
State University of New York, Buffalo, NY	M.A.	1974	Biology
State University of New York, Buffalo, NY	M.D.	1976	Medicine
State University of New York, Buffalo, NY	Ph.D.	1978	Cell & Mol. Biol

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

**Professional Experience:**

- 7/79 - 6/86 Assistant Professor, Department of Pathology, University of Colorado Health Sciences Center  
 7/86 - Associate Professor, Department of Pathology, University of Colorado Health Sciences Center  
 9/87 - Associate Professor, Department of Urology, University of Colorado Health Sciences Center  
 10/83 - Director, Laboratory of Histology and Immunohistochemistry, Department of Pathology, University of Colorado Health Sciences Center  
 3/88 - Director, Pathology Core Laboratory for Histopathology and Tissue Procurement, University of Colorado Health Sciences Center

**Honors and Awards:**

- 1981-1985 Collaborating Urologic Pathologist, Abbott Laboratories - Leuprolide Studies  
 1984-1986 Collaborating Urologic Pathologist, Syntex Research - Nafarelin Studies  
 1986-1989 Collaborating Urologic Pathologist, Roussel - Anandron Studies  
 1986-1990 Member, NCI Organ Systems Prostate Working Group, Subcommittees on Diagnostic Nomenclature and Research Resources  
 4/86 - Member, Southwest Oncology Group, Pathology Committee, Urology Committee, Genitourinary Pathology (Chairman)  
 3/87 - Editorial Board, Surgical Pathology  
 7/87 - Board of Directors, International Society of Differentiation  
 5/82 - Member, American Association of Pathologists  
 1989 Diplomate, American Board of Pathology, Anatomic Pathology  
 1990 - NIH/Pathology B Study Section

**Representative Publications: (Selected from over 50)**

- Gaeta, J.F., Asirwatham, J.E., Miller, G.J., Murphy, G.P. (1980) Histologic grading of primary prostatic cancer: A new approach to an old problem. *J. Urol.* 123: 689-693.  
 Miller, G.J., Runner, M.N., Chung, L.W.K. (1985) Tissue interactions and prostatic growth. II. Morphological and biochemical characterization of adult mouse prostatic hyperplasia induced by fetal urogenital sinus implants. *The Prostate* 6: 241-253.  
 Miller, G.J., Ferrara, J.A. (1988) Identification of species origin in normal and neoplastic mesenchymal cells. *Stain Tech.* 63: 15-21.  
 Miller, G.J., DeMarzo, A.M. (1988) Ultrastructural localization of matrix vesicles and alkaline phosphatase in the Swarm rat chondrosarcoma: Their role in cartilage calcification. *Bone* 9: 235-241.

- Miller, G.J. (1988) Stage A prostate cancer in the People's Republic of China. In *A Multidisciplinary Analysis of Controversies in the Management of Prostate Cancer* (Coffey, D.S., Resnick, M., Door, A, Karr, J.P., eds) Plenum, New York, NY, pp. 1724.
- Miller, G.J. (1989) Histopathology of prostate cancer: Prediction of malignant behavior and correlation with ultrasonography. *Urology* **33**: 18-26.
- Miller, G.J. (1989) Pathological aspects of prostate cancer: Prediction of malignant potential. *Urology* **34**: 5-9.
- Thrasher, J.B., Miller, G.J., Wettlaufer, J.N. (1990) Bladder leiomyosarcoma following cyclophosphamide therapy for lupus nephritis. *J. Urol.* **143**: 119-121.
- Miller, G.J. (1990) New developments in the grading of prostatic carcinoma. *Seminars in Urology* **8**: 9-18.
- Thickman, D., Miller, G.J., Hopper, K.D., Raife, M. (1990) Prostate cancer: Comparison of pre-operative 0.35T MRI with whole-mount histopathology. *Magnetic Resonance Imaging* **8**: 205-211.
- Orlicky, D.J., Miller, G.J., Evans, R.M. (1990) Identification and purification of a bovine corpora luteal membrane glycoprotein with [3H]prostaglandin F2alpha binding properties. *Prostaglandins, Leukotrienes and Essential Fatty Acids* **41**: 51-61.
- Lile, R., Thickman, D., Miller, G.J., Crawford, E.D. (1990) Prostatic comedocarcinoma: Correlations of sonograms with pathologic specimens in three cases. *Am. J. Roent.* **155**: 303-306.
- Dienhart, D.G., Schmelter, R.F., Lear, J.L., Miller, G.J., Glenn, S., Bloedow, D.C., Kasliwal, R., Moran, P. Seligman, P., Murphy, J.R., Kortright, K., Bunn, P.A., Jr. (1990) Imaging of non-small cell lung cancers with a monoclonal antibody, KC4G3, which recognizes a human milk fat globule antigen. *Cancer Res.* **50**: 7068-7076.
- Kantorowitz, D., Miller, G.J., Ferrara, J.A., Ibbott, G.S., Fisher, R., Ahrens, C.R. (1990) Preoperative versus postoperative irradiation in the prophylaxis of heterotopic bone formation in rats. *Int. J. Radiat. Oncol. Biol. Phys.* **19**: 1431-1438.
- Popek, E.J., Tyson, R.W., Miller, G.J., Caldwell, S.A. (1991) Prostate development in prune belly syndrome (PBS) and posterior urethral valves (PUV): Etiology of PBS - Lower urinary tract obstruction or primary mesenchymal defect? *Pediatr. Pathol.* **11**: 1-29.
- Miller, G.J., Stapleton, G.E., Houmiel, K.L., Ferrara, J.A. (1991) Specific receptors for vitamin D3 in human prostatic carcinoma cells. In: *Molecular and Cellular Biology of Prostate Cancer* (Karr, J.B., Coffey, D.S., Smith, R.G., Tindall, D.J., eds) Plenum, New York, NY, pp. 253-258.
- Merz, V.W., Miller, G.J., Kadman, D., Park, S., Egawa, S., Scardino, P.T., Thompson, T.C. (1991) Elevated TGF-beta1 mRNA levels are associated with ras and myc-induced carcinomas in reconstituted mouse prostate: Evidence for a paracrine role during progression. *Mol. Endocrinol.* **5**: 503-513.
- Donohue, R.E., Miller, G.J. (1991) Adenocarcinoma of the prostate: Biopsy to whole-mount. *Urol. Clin.* **18**: 449-452.
- Egawa, S., Kadmon, D., Miller, G.J., Scardino, P.T., Thompson, T.C. (1992) Alterations in mRNA levels for growth-related genes after transplantation into castrated hosts in oncogene-induced clonal mouse prostate carcinoma. *Mol. Carcinogenesis* **5**: 1-10.
- Miller, G.J., Stapleton, G.E., Ferrara, J.A., Lucia, S., Pfister, S., Hedlund, T.E., Upadhyay, P. (1992) The human prostate carcinoma cell line LNCaP expresses biologically active, specific receptors for 1alpha, 25-dihydroxyvitamin D3. *Cancer Res.* **52**: 515-520.



**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME M. Allen NORTHRUP	POSITION TITLE Principal Engineer		
EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
University of Vermont, Burlington, VT	B.A.	1976	Zoology
California State University, Arcata, CA	M.A.	1981	Biology
University of California, Davis, CA	Ph.D.	1991	Biomed. Engineering
Lawrence Livermore National Laboratory, CA	Postdoc.	1992	Engineering Research

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

1981-1986 Engineer, Finnigan Corporation, Mass Spectrometry Instrumentation Development  
 1987-1990 Graduate Fellow, Lawrence Livermore National Laboratory, Biomedical and Environmental Research Program  
 1991-Present Principal Engineer, Lawrence Livermore National Laboratory, Engineering Research Division  
 1991-Sept. 1993 Visiting Scholar, University of California at Berkeley, Electrical Engineering and Computer Science Department  
 Sept. 1993-Present Adjunct Assistant Professor, University of California at San Francisco Medical Center, Radiology Department

*Honors and Awards:*

NSF-NATO Advanced Study Institutes Travel Awards : Biological Spectroscopy, Loutraki, Greece, 1988; and Convective Heat and Mass Transfer in Porous Media, Izmir, Turkey, 1990.  
 Graduate Fellowship: Associated Western Universities/U.S. Department of Energy, 1988, 1990-91.  
 National Research Council Postdoctoral Fellowship, 1991, (not accepted)

*Publications:*

Northrup, M. A., Evidence for the Detoxification of Methylmercury by Harbor Seals, *Phoca vitulina richardi*. (poster and abstract) Fourth Biennial Conf. Biology of Marine Mammals, San Francisco, CA, Dec., 1980.  
 Schmidt, M. and Northrup, M. A., A Cost-Effective Instrument for Accurate Trace Element Analysis. *Amer. Lab.*, February, 1986, 125-134.  
 Northrup, A., Computer-Aided Chemistry: Effective Management of Chemical Information. *Biotechnology*, 5 (5), 1987, 455-459.  
 Northrup, M. A., Stanker, L., Vanderlaan, M., and Watkins, B., Development and Characterization of a Fiber Optic Immuno-Biosensor. In *Spectroscopy of Inorganic Bioactivators*, T. Theophanides (Ed.), Kluwer Academic, Norwell, 1989, 229-241.

- Northrup, M. A., Langry, K., and Angel, S. M., Stability Studies of a pH-Sensitive Polymer Matrix: Applications to Fiber Optic pH Sensors. *Proceedings of SPIE Conference on Optical Fibers in Medicine V*, vol. 1201, 1990, 368-374.
- Northrup, M. A., Kulp, T.J., and Angel, S.M., Fluorescent Particle Imaging Velocimetry: Applications to Porous Media Flow. *Applied Optics*, vol. 30, (1991), 3034-3040.
- Northrup, M. A., Kulp, T.J., Angel, S.M., and Pinder, G.F., Determination of Velocity Vectors in Porous Media With Fluorescent Particle Image Velocimetry (FPIV). In *Convective Heat and Mass Transfer in Porous Media*. S. Kakac, B. Kilkis, F. Kulacki, and F. Arinc (Eds.), Kluwer Academic Pub., Dordrecht, The Netherlands, (1991), 867-881.
- Northrup, M.A., Kulp, T.J., and Angel, S.M., Application of Fluorescent Particle Imaging to Measuring Flow in Complex Media. *Analytica Chimica Acta*, vol. 255, no. 2, (1991), 275-282.
- Northrup, M.A., Kulp, T.J., and Angel, S.M., Imaging of Interstitial Velocity Fields and Tracer Distributions in a Refractive Index-Matched Porous Medium. *International Seminar on Heat and Mass Transfer in Porous Media*, Dubrovnik, Yugoslavia, May, 1991.
- Northrup, M.A., Kulp, T.J., and Angel, S.M., Measurement of Interstitial Velocity Fields and Tracer Distributions in a Porous Medium—Preliminary Spatial Averaging of Velocity Fields. submitted to *Water Resources Research*, October, 1992.
- Anderson, B.L., Northrup, M.A., Langry, K., and Angel, S.M., Fiber-Optic Fluorescence-Based Reversible Sensors for the Remote Detection of Trace Levels of Organic Vapors. 1993. In preparation.
- Ching, M., Northrup M.A., and White, R.M., Biochemical Microflow Systems. Industrial Advisory Board Reports, Berkeley Sensor and Actuator Center, University of California, Berkeley, Oct. 1991, and March, 1992.
- Northrup M.A., Ching, M., and White, R.M., Microfabricated Biochemical Reactor: Amplification of DNA by the Polymerase Chain Reaction (PCR). Presented at Industrial Advisory Board Meeting, Berkeley Sensor and Actuator Center, University of California, Berkeley, Oct. 1992.
- Northrup, M.A., Kulp, T.J., Angel, S.M. and G.F. Pinder, Direct Measurement of Interstitial Velocity Field Variations in a Porous Medium using Fluorescent-Particle Image Velocimetry. *Chemical Engineering Science*, vol. 48, no. 1, (1993), 13-21.
- Northrup, M.A., Ching, M.T., White, R.M., and Watson, R.T., DNA Amplification in a Microfabricated Reaction Chamber. In *Transducers '93, Seventh International Conference on Solid State Sensors and Actuators*, Yokohama, Japan, June 7-10, 1993.
- Angel, S.M. and Northrup, M.A., Stability of a Fiber Optic pH Sensor at 100 °F. Electric Power Research Institute (EPRI), Report TR-101972, Feb. 1993.

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME	POSITION TITLE
Michael P. OSBORNE	Director, Strang Cancer Prevention Center

EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
University of London, UK	M.B./M.S.	1970	Medicine & Surgery
Royal College of Surgeons of England	F.R.C.S.	1975	Surgery
University of London	M.S.	1980	Surgery
American College of Surgeons	F.A.C.S.	1984	Surgery

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

1970- 1971 House Physician, West London Hospital, (Charing Cross Group of Hospitals)  
 1971- 1972 House Surgeon to the Professorial, Dept Surgery, (Charing Cross Group of Hospitals)  
 1972- 1973 Senior House Officer, Accident and Emergency Surgery, West Middlesex Hospital  
 1972- 1973 Senior House Officer, General Surgery and Urology, St. James Hospital, London  
 1973- 1974 Resident Surgical Officer, Cardiothoracic Surgery, Brompton Hospital, London  
 1973- 1976 Registrar, General Surgery, Orthopedics and Trauma, West Herts Hospital  
 1976- 1977 Registrar, General Surgery, Surgical Oncology, Charing Cross Hosp., London  
 1977- 1978 Lecturer in Surgery & Wellcome Trust Fellow, Charing Cross Hosp. Med. School, Univ. of London  
 1978- 1980 Honorary Lecturer and Sr Registrar, The Royal Marsden Hosp. and Inst. of Cancer Research, London  
 1980- 1981 Fellow, Surgical Oncology, Dept. of Surgery, Memorial Sloan-Kettering Cancer Center (MSKCC)  
 1981- 1984 Assist. Prof. of Surgery, Cornell University Medical Center (CUMC)  
 1981- 1990 Asst. Attending Surgeon, Memorial Hospital for Cancer and Allied Diseases  
 1982- 1989 Visiting Assoc. Physician, The Rockefeller University Hospital  
 1982- 1989 Adjunct Faculty Member, The Rockefeller University  
 1984- 1990 Assistant Member, MSKCC  
 1984- 1991 Associate Professor of Surgery, CUMC  
 1985- 1991 Head, Breast Cancer Research Laboratory, MSKCC  
 1990- 1991 Associate Member, MSKCC  
 1990- 1991 Associate Attending Surgeon, MSKCC  
 1986- Director, High Risk Program, Preventive Medicine Institute/Strang Clinic  
 1991- Consultant, Breast Service, Department of Surgery, MSKCC  
 1991- Visiting Physician, The Rockefeller University Hospital  
 1991- Director and Chief Executive Officer, Strang Cancer Prevention Center  
 1991- Chief, Breast Service, New York Hospital-Cornell Medical Center  
 1991- Professor of Surgery with Tenure, CUMC

*Honors and Awards:*

Thomas Henry Green Prize in Clinical Medicine  
 William Travers Prize in Obstetrics and Gynecology  
 David Winsor Essay Prize  
 The Golding Medal (The Governor's Clinical Gold Medal)

Raven Prize, British Association of Surgical Oncologists  
Llewelyn Scholarship  
Gosse Scholarship, Charing Cross Hosp Med School, Univ of London  
Norman C. Lake Prize in Surgery

*Representative Publications:*

- Osborne MP, Asina S, Wong GY, Old LJ, Cote RJ, Rosen PP. Sensitivity of immunocytochemical detection of breast cancer cells in human bone marrow. *Cancer Res.* 1991;51:2706-2709.
- Cote RJ, Rosen PP, Lesser ML, Old LJ, Osborne MP. Prediction of early relapse in patients with operable breast cancer by detection of occult bone marrow micrometastases. *J. Clin. Oncol.* 1991;9:1749-1756.
- Narayanan R, Lawler KG, Schaapveld RQJ, Cho KR, Vogelstein B, Tran PBV, Osborne MP, Telang NT. Antisense RNA to the putative tumor suppressor gene DCC transforms RAT-1 fibroblasts. *Oncogene* 1992;7:553-56.
- Osborne MP, Borgen PI, Wong GY, Rosen PP, McCormick B. Salvage mastectomy for local-regional relapse after breast conserving surgery and radiation therapy. *Surg. Gynecol. Obstet.* 1992;174:189-194.
- Osborne MP, Ruperto JF, Crowe JP, Rosen PP, Telang NT. The effect of tamoxifen on preneoplastic cell proliferation in N-nitroso-N-methylurea-induced mammary carcinogenesis. *Cancer Res.* 1992;52:1477-1480.
- Telang NT, Suto A, Wong GY, Osborne MP, Bradlow HL. Induction by estrogen metabolite 16 $\mu$ -hydroxestrone of genotoxic damage and aberrant proliferation in mouse mammary epithelial cells. *J. Natl. Cancer Inst.* 1992;84:634-636.
- Telang NT, Bradlow HL, Osborne MP. Molecular and endocrine biomarkers in non-involved breast: relevance to cancer chemoprevention. *Journal of Cellular Biochemistry* 1992;16:161-169.
- Borgen PI, Vlamis V, Potter C, Hoffman B, Wong GY, Kinne DW, Osborne MP, McKinnon WMP. Current management of male breast cancer: A review of 104 cases. *Annals of Surgery* 1992;215:451-459.
- Yahalom J, Petrek JA, Biddinger PW, Kessler S, Dershaw DD, McCormick B, Osborne MP, Rosen PP. Breast cancer in patients irradiated for Hodgkins disease: A clinical and pathological analysis of 45 events in 37 patients. *Journal of Clinical Oncology* 1992;10:1674-1681.
- Tiwari RK, Mukhopadhyay B, Osborne MP. Desensitization of human breast cancer cells by interferon- $\mu$ . *International Journal of Oncology* 1992;1:601-605.
- Dershaw DD, McCormick B, Osborne MP. Detection of local recurrence after conservation therapy for breast carcinoma. *Cancer* 1992;70:493-496.
- Breuer B, Kash K, Rosenthal G, Diemer K, Osborne MP, Miller DG. Reporting bilaterality status in first-degree relatives with breast cancer: A validity study. *Genetic Epidemiology* 1993; 10:245-256.
- Osborne MP, Bradlow HL, Wong GY, Telang NT. Upregulation of Estradiol C16 alpha-Hydroxylation in breast tissue: A potential biomarker of breast cancer risk. *JNCI* 1993;85:1917-1920.
- Tiwari RK, Li Guo, Bradlow HL, Telang NT, Osborne MP. Selective responsiveness of human breast cancer cells to indole-3-carbinol, a chemopreventive agent. *JNCI* 1994;86:126-131.

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

<b>NAME</b> David H. PERSING	<b>POSITION TITLE</b> Senior Associate Consultant /Assistant Professor		
<b>EDUCATION</b> (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
<b>INSTITUTION AND LOCATION</b>	<b>DEGREE</b>	<b>YEAR CONFERRED</b>	<b>FIELD OF STUDY</b>
San Jose State University, San Jose, CA	B.S.	1979	Biochemistry
University of California, San Francisco, CA	Ph.D.	1988	Biochem. & Biophys.
Yale School of Medicine, New Haven, CT	M.D.	1988	Medicine
Yale School of Medicine, New Haven, CT	Residency	1988-90	Pathology

**RESEARCH AND/OR PROFESSIONAL EXPERIENCE:** Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. **DO NOT EXCEED TWO PAGES.**

*Professional Experience:*

Dr. Robert Fowler, Department of Biology, San Jose State University, Spring 1979-Summer 1980  
 "Mutational specificity of the base analogue, 2-aminopurine in *Escherichia coli*."  
 Medical Scientist Training Program, University of California, San Francisco, October 1980-June 1988  
 Dr. Phillip Coffino, Department of Microbiology, UC San Francisco, Summer 1980-Spring 1981  
 "Mechanism of 2-aminopurine mutagenesis in mouse T-lymphosarcoma cells."  
 Dr. Harold E. Varmus, Department of Microbiology, UC San Francisco, Summer 1981-Fall 1981  
 "Molecular analysis of mutant src alleles."  
 Dr. Patrick O'Farrell, Department of Biochemistry, UC San Francisco, Summer 1982-Winter 1983  
 "Isolation and characterization of DNA clones representing the engrailed locus of *Drosophila melanogaster*."  
 Graduate Program in Biochemistry and Biophysics, Division of Genetics, University of California, San Francisco. September 1982-March 1987  
 Drs. Donald Ganem and Harold Varmus (thesis advisors), Department of Biochemistry, and Biophysics, UC San Francisco, Spring 1983 to February 1987  
 "Identification and characterization of the presurface proteins of hepatitis B virus."  
 Resident and Fellow, Department of Laboratory Medicine, Yale-New Haven Hospital, July 1988-June 1990.  
 Dr. Ari Helenius, Department of Cell Biology, Yale University School of Medicine, January 1989-August 1990  
 "Papovavirus assembly, disassembly and cellular interactions"  
 Senior Associate Consultant, Department of Laboratory Medicine and Experimental Pathology, and  
 Assistant Professor, Mayo Foundation, September 1990-present

*Honors and Awards:*

Graduation Magna Cum Laude, San Jose State University, 1979  
 Departmental Honors in Chemistry, 1979  
 Finalist, Dean's Prize for Research, School of Medicine, 1981  
 Graduate Dean's prize for Student Research University of California, San Francisco, 1984  
 Gip A. Hudson and James A. McLaughlin Awards, National Student Research Forum, Galveston, Texas, 1985  
 University Patent Funds Award, 1986  
 Young Investigator Award, Academy of Clinical Laboratory Physicians and Scientists, 1990  
 Mayo Foundation Scholar, 1991  
 Editorial board, Journal of Clinical Microbiology, 1990-1993

*Representative Publications:* (out of 49 total)

- Hunt, J.M., A. Telenti, G.D. Roberts, L. Stockman, T.A. Felmlee, and D.H. Persing. (1993) Detection of a genetic locus encoding resistance to rifampin in mycobacterial cultures and in clinical specimens. Submitted for publication.
- Thomford, J.W., P.A. Conrad, S.R. Telford, D. Mathiesen, B.H. Bowman, A. Spielman, M.L. Eberhard, B.L. Herwaldt, R.E. Quick, and D.H. Persing. (1993) A newly identified piroplasm of humans. Submitted for publication.
- Persing, D.H., C. Glaser, P. Krause, J. Thomford, and P.A. Conrad. (1993) A new tick-transmitted zoonotic piroplasm found in the Western United States. Submitted for publication.
- Rys, P.N., and D.H. Persing. (1993) Preventing false positives: quantitative evaluation of three protocols for inactivation of polymerase chain reaction amplification products. *J. Clin. Microbiol.* 31(9):2356-2360.
- Persing, D.H., D. Mathiesen, W.F. Marshall, S.R. Telford, A. Spielman, J.W. Thomford, and P.A. Conrad. (1992) Detection of *Babesia microti* by polymerase chain reaction. *J. Clin. Microbiol.* 30:2097-2103.
- Shapiro, E., M. Gerber, N.B. Holabird, A.T. Berg, H.M. Feder, Jr., G.L. Bell, P.N. Rys, and D. Persing. (1992) A controlled trial of antimicrobial prophylaxis for Lyme disease after deer-tick bites. *N. Engl. J. Med.* 327:1769-1773.
- Malawista, S.E., T.L. Moore, D.E. Dodge, T.J. White, R.T. Schoen, and D.H. Persing. (1992) Failure of multitarget detection of *B. burgdorferi*-associated DNA sequences in synovial fluids of patients with juvenile rheumatoid arthritis: a cautionary note. *Arthritis and Rheumatism* 35:246-247.
- Armstrong, A.L., S.W. Barthold, D.H. Persing, and D.S. Beck. (1992) Carditis in Lyme disease susceptible and resistant strains of laboratory mice infected with *Borrelia burgdorferi*. *Am. J. Trop. Med. Hyg.* 47(2):249-258.
- Conrad, P.A., J. Thomford, A. Marsh, S.R. Telford III, J. Anderson, A. Spielman, E. A. Sabin, I. Yamane, and D.H. Persing. (1992) Ribosomal DNA probe for differentiation of *Babesia microti* and *B. gibsoni* isolates. *J. Clin. Microbiol.* 30:1210-1215.
- Fikrig, E., S.W. Barthold, D.H. Persing, X. Sen, F.S. Kantor, and R.A. Flavell. (1992) *Borrelia burgdorferi* strain 25015: characterization of *OspA* and vaccination against infection. *J. Immunol.* 148:2256-2260.
- Persing, D.H. (1991) The polymerase chain reaction: Trenches to benches. *J. Clin. Microbiol.* 29:1281-1285.
- Persing, D.H., S.R. Telford, III, A. Spielman, and S.W. Barthold. (1990) Detection of *Borrelia burgdorferi* infection in *Ixodes dammini* ticks using the polymerase chain reaction. *J. Clin. Microbiol.* 28:566-572.
- Persing, D.H., S.R. Telford, III, P.N. Rys, D.E. Dodge, T.J. White, S.E. Malawista, and A. Spielman. (1990) Detection of *Borrelia burgdorferi* DNA in museum specimens of *Ixodes dammini* ticks. *Science* 249: 1420-1423.
- Persing, D.H., H.E. Varmus, and D. Ganem. (1987) The preS1 protein of hepatitis B virus is acylated at its N-terminus with myristic acid. *J. Virol.* 61:1672-1677.
- Persing, D.H., H.E. Varmus, and D. Ganem. (1986) Inhibition of secretion of hepatitis B surface antigen by a related presurface polypeptide. *Science* 234:1388-1391.

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME Susan P. PROUDFOOT		POSITION TITLE Executive Director Procurement Systems Manager	
EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
University of Denver, CO	B.A.	1977	Economics
University of Houston, Houston, TX	M.S.H.A.	1985	Health

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

1977 - 79 Planning Associate, St. Anthony Hospital Systems, Denver, CO  
 1979 - 81 Senior Corporate Planner, Mercy Hospital, Miami, FL  
 1981 - 84 Senior Management Engineer, St. Lukes Episcopal Hospital, Texas Children's Hospital, Texas Heart Institute (a tripartite facility), Houston, TX  
 1985 Senior Consultant, Deloitte, Haskins & Sells, Denver, CO  
 1985 - 87 State Administrative Director (Colorado Case Mix Project Officer), Colorado Department of Social Services, Denver, CO  
 1987 - 89 Vice President, Center for Health Policy Research, Denver, CO  
 1989 - Executive Director, Lung Cancer Institute of Colorado, Denver, CO

*Professional Distinctions:*

Faculty, Greater Houston Management Systems Society's 6th Annual Conference, June, 1983.  
 Guest Speaker, Greater Houston Management systems Society Quarterly Meeting, Winter, 1984;  
 Lecture: Management Engineers and Hospital Planners - A Meeting of the Minds.  
 Guest Lecturer, University of Houston, Administration of Health Services Graduate Program, October, 1984;  
 Lecture: Performance of Staffing Studies in Hospital Departments - The Required Elements.  
 Guest Lecturer, St. Francis College, Administration of Health Services Graduate Program, July, 1985;  
 Lecture: Devising Work Standards for Productivity Measurement.  
 Faculty, Colorado Hospital Association Annual Conference, August, 1985.  
 Subcommittee Chairperson, Colorado Governors Conference on Aging, October, 1987. (Selected by Colorado Commission on Aging to serve as Chairperson of Long-Term Care Cost and Quality Issue Subcommittee for Conference)  
 Guest Lecturer, University of Colorado, Administration of Health Services Graduate Program, October, 1988;  
 Lecture: Technical Components of Case Mix Reimbursement Systems.  
 Guest Lecturer, University of Colorado, Administration of Health Services Graduate Program, Dec. 1988;  
 Lecture: Political Aspects of Implementing a Medicaid Nursing Home Case Mix Reimbursement Project.  
 Speaker, Colorado Association of Homes and Services for the Aging 22nd Annual Meeting, June, 1989.  
 Lecture: Case Mix Reimbursement Developments in Colorado.

*Bibliography:*

- Proudfoot, S.P. (1986) Legislative Report: Colorado case mix reimbursement pilot program: Status report for Colorado General Assembly. Denver, CO, Bureau of Medical Services, Colorado Department of Social Services.
- Proudfoot, S.P. (1987) Legislative Report: Colorado case mix reimbursement program: Status report for Colorado General Assembly. Denver, CO, Bureau of Medical Services, Colorado Department of Social Services.
- Shaughnessy, P., Proudfoot, S., and Kramer, A. (1988) Study Design and Planning Phase Progress Report, Study Paper 1. Denver, CO: Center for Health Policy Research (HCFA Cooperative Agreement 17-C-98971/9-01).
- Butler, P.A., and Proudfoot, S.P. (1988) Home Health Care Quality Assurance, Study Paper 2. Denver, CO: Center for Health Policy Research (HCFA Cooperative Agreement 17-C-98971/9-01).
- Butler, P.A., and Proudfoot, S.P. (1988) Medicare Home Health Care Reimbursement Issues, Study Paper 4. Denver, CO: Center for Health Policy Research (HCFA Cooperative Agreement 17-C-98971/9-01).
- Butler, P.A., and Proudfoot, S.P. (1988) HMOs and Home Health Care Under Medicare, Study Paper 5. Denver, CO: Center for Health Policy Research (HCFA Cooperative Agreement 17-C-98971/9-01).
- Butler, P.A., and Proudfoot, S.P. (1989) Third Party Payment for Community-Based Long-Term Care. Study Paper 6. Denver, CO: Center for Health Policy Research (HCFA Cooperative Agreement 17-C-98971/9-01).
- Schlenker, R.E., and Proudfoot, S.P. (1988) Resident Assessment Features and Issues, Briefing Paper 1A, Center for Health Services Research, University of Colorado Health Sciences Center, Denver, Colorado.
- Schlenker, R.E., Carter, D., Stiles, J., Proudfoot, S.P. (1988) Recommended Resident Classification System, Briefing Paper 1B, Center for Health Services Research, University of Colorado Health Sciences Center
- Proudfoot, S.P., and Schlenker, R.E. (1988) Quality Assurance, Briefing Paper 2, Center for Health Services Research, University of Colorado Health Sciences Center, Denver, Colorado.
- Schlenker, R.E., Stiles, J., Proudfoot, S.P., Carlough, T. (1988) Proposed Rate Setting Methodology, Briefing Paper 3, Center for Health Services Research, University of Colorado Health Sciences Center, Denver, Colorado.
- Schlenker, R.E., and Proudfoot, S.P. (1989) Colorado Nursing Home Case Mix Reimbursement project Final Report, Center for Health Services Research, University of Colorado Health Sciences Center, Denver, Colorado.
- Schlenker, R.E., and Proudfoot, S.P. (1989) What Case-Mix Systems Can Do for Providers. Provider 15: 18-19.
- Kennedy, T.C., Petty, T.A., Piantadosi, S., Proudfoot, S.P., Saccomanno, G., Tockman, M.S.: Comparison of sputum collection techniques for cytologic evaluation in patients with airflow obstruction (submitted).



**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME Leonid L. REZNIKOV		POSITION TITLE Research Fellow	
EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Leningrad Med Inst of Hygiene, St. Petersburg, Russia	M.D.	1984	Medicine
Semasho Medical Institute, St. Petersburg, Russia	M.D.	1986	Andrology / Urooncology
Pediatric Medical Institute, St. Petersburg, Russia	Ph.D.	1990	Laser Medicine

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

- 1984 - 1986 Resident, Dept. of Urology and Andrology, State Postgraduate Medical Institute, St. Petersburg, Russia
- 1986 - 1989 Staff Urologist & Professional Research Assistant, Laser Surgery & Therapy Group, Pediatric Medical Institute, St. Petersburg, Russia
- 1989 - 1992 Assistant Professor of Surgical Urology & Senior Research Associate, Laser Medical Research Laboratory, Pediatric Medical Institute, St. Petersburg, Russia
- 1993 - Present Visiting Research Fellow, Ob/Gyn Dept., Univ. Colorado School of Medicine, Denver
- 1993 - Present Research Fellow, Molecular Genetics/Oncology Laboratory, Pathology Department, The Children's Hospital & University of Colorado Cancer Center, Denver, CO

*Honors and Awards:*

- 1984 Honorable M.D. degree
- 1990 Fulfillment with early completion, Ph.D. degree, Study Prize
- 1991 Winner of All-Russian competition of Scientific Projects

*Representative Publications:*

- Reznikov, L. Ya., Glezer, Ya.V., & Reznikov, L.L.: The usage of apparatus "Real" for Urology. *Meditinskaya tekhnika*. 2:51-54, 1989.
- Reznikov, L.L., Zus, B.A.: The influences of low-intensity laser radiation of different spectral range on spermatogenic glands. In *Hygienic and Medical-biological Aspects of Public Health*, V.Ignatuk, Ed., Leningrad, LSGMI, pp. 104-107, 1989.
- Reznikov, L.L., Reznikov, L.Ya., Murzin, A.G.: On the possible model of investigation of using low-intensity laser radiation. In *Model Systems in Medical and Biochemical Study*. V.A. Dadaly, Ed., Leningrad, LSGMI, pp. 91-94, 1989.
- Miroshniyov, B.I., Yakushev, V.I., Vasiliev, A.I., Reznikov, L.L.: Experience in using sound stimulation of upper urinary ducts and medical treatment of ureterolithiasis. *Vestnik khirurgii*. 2:128-130, 1990.
- Miroshniyov, B.I., Reznikov, L.L., Yakushev, V.I.: Experience in compound treatment of chronic prostatitis. *Voprosy kurortologii*. 3:38-40, 1990.
- Reznikov, L.L., Miroshniyov, B.I.: Laser therapy of acute nonspecific epididymitis. *Vestnik khirurgii*. 10: 121-123, 1990.

- Reznikov, L.Ya., Arkhangel'skaya, Ye.I., Reznikov, L.L.: Experience in medical treatment of residual urethritis and its complications by low-intensity laser radiation. *Vestnik dermatologii i venerologii*. 11:36-39, 1990.
- Reznikov, L.L., Miroshnikov, B.I., Zaychik, A.Sh., Yakushev, V.I.: Laser therapy in cases of acute nonspecific epididymo-orchitis. *Urol. Nephrol.* 2:45-49, 1990.
- Reznikov, L.L., Soms, L.N., Murzin, A.G., Supilko, S.V., Voskresenskaya, Yu.A., Khachatryan, S.A.: The influence of permanent low-intensity laser radiation on technical and medical personnel. In *Scientific Articles of LSGMI*, V. Ignatuk, Ed., Leningrad, LSGMI, pp. 88-90, 1990.
- Miroshnikov, B.I., Reznikov, L.L., Ogurtsov, R.P., Khachatryan, S.A.: Immunological problems of different modes of treatment of acute nonspecific epididymitis. *Vestnik khirurgii*. 2:156-160, 1992.
- Reznikov, L.Ya., Reznikov, L.L.: Low-intensity laser radiation in the treatment of sexual and urinary diseases. In *Laser in Biology and Medicine*, L. Zazulevskaya, Ed., Alma-Ata, pp. 75-83, 1992.
- Reznikov, L.L., Pavlova, R.N., Murzin, A.G., Boiko, V.N., Pupkiova, L.S., Soms, L.N.: On the similarity between the mechanism of soft-laser radiation and chemical adaptogen action. Proceedings of Low-Energy Laser Effects on Biological Systems. *SPIE Proceeding Series* 1883:91-98, 1993.

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

<b>NAME</b> Basil RIGAS	<b>POSITION TITLE</b> Associate Professor
----------------------------	--

<b>EDUCATION</b> (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
<b>INSTITUTION AND LOCATION</b>	<b>DEGREE</b>	<b>YEAR CONFERRED</b>	<b>FIELD OF STUDY</b>
Athens University Medical School	M.D.	1972	Medicine
Athens University Medical School	Dr.Md.Sci.	1975	Biochemistry

**RESEARCH AND/OR PROFESSIONAL EXPERIENCE:** Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

- 1992-present: Associate Professor of Medicine and Microbiology, Cornell University Medical College, New York, NY; Acting Chief, Division of Digestive Diseases, The New York Hospital, New York, NY
- 1986-1992: Assistant Professor of Medicine, Cornell University Medical College, New York, NY; Attending Physician, Division of Digestive Diseases, The New York Hospital, New York, NY
- 1991-1992: Assistant Professor of Microbiology, Cornell University Medical College, New York, NY
- 1985-1986: Associate Research Scientist, Department of Human Genetics, Yale University School of Medicine, New Haven, CT
- 1983-1985: Postdoctoral Fellow, Division of Digestive Diseases and Department of Human Genetics, Yale University School of Medicine, New Haven, CT
- 1980-1983: Postdoctoral Fellow, Department of Biochemistry, Brandeis University, Waltham, MA.
- 1974-1977: Internship/Residency, Department of Medicine, Brown University Providence, R.I.

**SPECIALTY CERTIFICATION**

American Board of Internal Medicine; Diplomate in Gastroenterology

**GRANTS & AWARDS**

Argall L. and Anna G. Hull Fund Cancer Research Award (1986)  
 Biomedical Research Support Grant from USPHS (1987)  
 Wendy Will Case Cancer Fund (1989)  
 Cystic Fibrosis Foundation (1989)  
 American Cancer Society (1990)  
 NIH, Human Genome Project (1991)  
 Ascher-Globus Teaching Award (1991)

**PATENTS**

1. RecA nucleoprotein filament and methods, No 4,888,274
2. A method for detecting the presence of anomalies in biological tissues and cells in natural and cultured form by infrared spectroscopy, No 5,038,039
3. A method for detecting the presence of anomalies in exfoliated cells using infrared spectroscopy, No 168,162

**SELECTED PUBLICATIONS:** (out of 38)

1. Rigas B, Van Vunakis H, Levine L. The effect of phenothiazines and their metabolites on prostaglandin production by rat basophilic leukemia cells in culture. *Prostagland Med.* 1981; 7:183-1035
2. Rigas B, Lewis RA, Austen KF, Corey EJ, Levine L. Identification and quantitation of arachidonic acid metabolic products in rabbit, rat and human saliva. *Arch Oral Biol.* 1983; 28:1031-1035
3. Rigas B, Welcher AA, Ward DC, Weissman SM. Rapid plasmid library screening using RecA-coated biotinylated probes. *Proc Natl Acad Sci USA.* 1986; 83:9591-9595
4. Lawrance SK, Srivastava R, Rigas B, Chorney MJ, Gillespie GA, Smith CL, Cantor CR, Weissman SM. Molecular approaches to characterization of megabase regions of DNA: Applications to the human major histocompatibility complex. *Cold Spring Harbor Symp Quant Biol.* 1986; 51:123-130
5. Rigas B, Morgelo S, Goldman IS, Wong PTT. Human colorectal cancers display abnormal Fourier-transform infrared spectra. *Proc Natl Acad Sci USA*, 1990, 87:8140-8144
6. Rigas B. Oncogenes and supressor genes: their involvement in colon cancer. *J Clin Gastroenterol*, 1990, 12: 494-499
7. McDougall CJ, Ngoi SS, Goldman IS, Godwin T, Felix J, DeCosse J, Rigas B. Reduced expression of HLA class I and II antigens in colon cancer. *Cancer Res* 1990, 50:8023-8027
8. Wong PTT, Rigas B. Infrared spectra of microtome sections of human colon tissues. *Appl Spectrosc* 1990, 44:1715-1718
9. Wong PTT, Papavassiliou ED, Rigas B. The phosphodiester stretching bands in the infrared spectra of human tissues and cultured cells. *Appl Spectrosc*, 1991, 45:1563-1567
10. Wong PTT, Wong RK, Caputo TA, Godwin TA, Rigas B. Infrared spectroscopy of exfoliated human cervical cells: Evidence of extensive structural changes during carcinogenesis. *Proc Natl Acad Sci USA*, 1991, 88:10988-10992
11. Rigas B, Wong PTT. Human colon adenocarcinoma cell lines display infrared spectroscopic features of malignant colon tissues. *Cancer Res*, 1992, 52:84-88
12. Tsioulis G, Godwin T, Goldstein M, Ngoi SS, McDougall CJ, DeCosse J, Rigas B. Loss of colonic HLA antigens in familial adenomatous polyposis. *Cancer Res*, 1992, 52:3449-3452
13. Wong PTT, Goldstein SM, Grekin RC, Godwin TA, Pivik C, Rigas B. Distinct infrared spectroscopic patterns of human basal cell carcinoma of the skin. *Cancer Res*, 1993, 53:762-765
14. Tsioulis GJ, Triadafilopoulos G, Goldin E, Rizo S, Papavassiliou ED, Rigas B. Expression of HLA Class I antigens in sporadic adenomas and histologically normal mucosa of the colon. *Cancer Res*, 1993, 53:2374-2378
15. Rigas B, Goldman IS, Levine L. Altered eicosanoid levels in human colon cancer. *J Lab Clin Med*, in press.

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME Saul J. SILVERSTEIN	POSITION TITLE Professor		
EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Cornell University, Ithaca, NY	B.S.	1968	Biology
Univ. of Florida, Gainesville, FL	Ph.D.	1971	Microbiology
Univ. of Chicago, Chicago, IL	Postdoc.	1974	Virology

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

Postdoctoral Fellow, Laboratory of Radiation Biology, The University of Florida	1971
Research Associate, Department of Microbiology, The University of Chicago	1971 - 1974
Assistant Professor, Department of Microbiology, Columbia University	1974 - 1980
Associate Professor, Department of Microbiology, Columbia University	1980 - 1987
Professor, Department of Microbiology, Columbia University	1987 - present
Vice-Director, Integrated Program in Cellular, Molecular and Biophysical Studies	1984 - 1987
Director, Integrated Program in Cellular, Molecular and Biophysical Studies	1987 - 1989
Visiting Professor, Japanese National Cancer Center	1987
Acting Chairman, Department of Microbiology	1989 - present
Deputy Director, Comprehensive Cancer Center, Columbia University	1990 - present

*Honors and Awards:*

N.Y. State Regents Scholarship	1964 - 1968
NSF Summer Fellow	1967
NIH Predoctoral Fellowship	1969 - 1971
Damon Runyon Memorial Postdoctoral Fellow	1971 - 1973
RCDA from USPHS, NCI	1978 - 1983

*Representative Publications:*

- Crum, C.P., Nuovo, G., Friedman, D. and Silverstein, S. Accumulation of RNA Homologous to Human Papillomavirus 16 Open Reading Frames in Genital Precancers. *J. Virol.* 62: 84, 1988.
- Nuovo, G., Crum, C.P. and Silverstein, S. Papillomavirus Infection of the Uterine Cervix. *Microbial Pathogenesis.* 3: 71, 1987.
- Gelman, I.H. and Silverstein, S. Dissection of Immediate Early Gene Promoters from Herpes Simplex Virus: Sequences that Respond to the Virus Transcriptional Activators. *J. Virol.* 61: 3167, 1987.
- Nuovo, G.J., Crum, C.P., de Villiers, E.-M., Levine, R. and Silverstein, S. Isolation of a Novel Human Papillomavirus (HPV-51) from a Cervical Condyloma. *J. Virol.* 62: 1452, 1988.
- Moy, R.L., Eliezri, Y.D., Nuovo, G.J., Zitelli, J.A., Bennet, R.G. and Silverstein, S. Squamous Cell Carcinoma of the Finger is Associated with Human Papillomavirus Type 16 DNA. *J.A.M.A.* 261: 2669, 1989.

- Zhu, X., Young, C.S.H. and Silverstein, S. An Adenovirus Vector Expressing Functional Herpes Simplex Virus ICP0. *J. Virol.* 62: 4544, 1988.
- del Angel, R.M., Papavassiliou, A.G., Fernandez-Tomas, Silverstein, S. and Racaniello, V.R. Multiple Protein Binding Sites Within the 5'-Untranslated Region of Poliovirus RNA. *Proc. Natl. Acad. Sci. USA.* 86: 8299, 1989.
- Papavassiliou, A.G. and Silverstein, S. Characterization of DNA-Protein Complex Formation in Nuclear Extracts with a Sequence from the Herpes Simplex Virus Thymidine Kinase Gene. *J. Biol. Chem.* 265: 1648, 1990.
- Papavassiliou, A.G. and Silverstein, S. Interaction of Cell and Virus Proteins with the Promoter/Regulatory and Leader Sequences of the Herpes Simplex Virus Thymidine Kinase Gene. *J. Biol. Chem.* 265: 9402, 1990.
- Gaitanaris, G.A., Papavassiliou, A.G., Rubock, P., Silverstein, S. and Gottesman, M.E. Renaturation of Denatured I Repressor Requires Heat-Shock Proteins. *Cell.* 61: 1013, 1990.
- Zhu, X., Chen, J. and Silverstein, S. Mutations in IE-0 Affect Reactivation of Herpes Simplex Virus in a Model Latency System. *J. Virol.* 64: 4489, 1990.
- Chen, J., Zhu, X. and Silverstein, S. Mutational Analysis of the Sequence Encoding ICP0 from Herpes Simplex Virus Type 1. *Virology*, 180: 207, 1991.
- Papavassiliou, A.G., Wilcox, K.W. and Silverstein, S. Phosphorylation of ICP4, the Major Regulatory Protein of Herpes Simplex Virus, Controls Differential Recognition of DNA Sequences. *EMBO J.*, 10: 397, 1991.
- Zhu, X., Chen, J. and Silverstein, S. Isolation and Characterization of a Functional cDNA Encoding ICP0 from Herpes Simplex Virus Type-1. *J. Virol.*, 65: 957, 1991.
- Flanagan, W.M., Papavassiliou, A.G., Rice, M., Hecht, L.B., Silverstein, S and Wagner, E.K. Analysis of the HSV-1 Promoter Controlling the Expression of U<sub>l</sub>38, a True Late Gene Involved in Capsid Assembly. *J. Virol.*, 65: 769, 1991.
- Zhu, X., Papavassiliou, A.G., Stunnenberg, H., and Silverstein, S. Expression of Herpes Simplex Virus Immediate Early Proteins ICP's 4 and 0 in a Vaccinia Virus Vector. *Virology*, 184: 67, 1991.
- Chen, J., Panagiotidis, C. and Silverstein, S. "Multimerization of ICP0, a Herpes Simplex Virus Immediate Early Protein". *J. Virol.* 66: 5598, 1992.
- Lungu, O., Sun, X.W., Felix, J., Richart, R.M., Silverstein, S. and Wright, T.C. Relationship of Human Papillomavirus Type to Grade of Cervical Intraepithelial Neoplasia. *J.A.M.A.* 267: 2493, 1992.
- Lungu, O. and Silverstein, S. A PCR Strategy that Differentiates Two Alleles for a Single Base Insertion Within a T Repeat. *Biotechniques*. 14: 4, 1992.
- Gershon, A., La Russa, P., Hardy, I., Steinberg, S., and Silverstein, S. Varicella Vaccine: The American Experience. *J. I. D.* 166: 63, 1992.
- Bohenzky, R., Papavassiliou, A.G., Gelman, I.H., and Silverstein, S. Identification of a Promoter Mapping Within the Reiterated Sequences that Flank the Herpes Simplex Virus Type 1 UL Region. *J. Virol.* 67: 632, 1993.

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME John J. SNINSKY		POSITION TITLE Senior Director of Research	
EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Bates College, Lewiston, Maine	B.S.	1972	Biology
Purdue University, Indiana	Ph.D	1976	Biology
Stanford University Schl. of Medicine, Stanford, CA	Post Doc	1980	Genetics and Medicine

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

**Professional Experience:**

1981-1984	Albert Einstein College of Medicine, Assistant Professor Dept. of Microbiology & Immunology, Molecular Biology
1984-Present	Albert Einstein College of Medicine, Visiting Professor
1984-1985	Cetus Corporation, Dept. of Microbial Genetics, Sr. Scientist
1985-1988	Cetus Corporation, Dept of Infectious Diseases, Director
1988-1991	Cetus Corporation, PCR Division, Sr. Director
1991-Present	Roche Molecular Systems, Inc., Senior Director, Research

**Honors and Awards:**

1972	Honors in Biology, Bates College
1973-1976	N.I.H. Traineeship Research
1977	Dean's Fellowship, Stanford University
1977-1979	American Cancer Society Postdoctoral Fellowship
1979-1980	Dean's Fellowship, Stanford University

**Representative Publications:** (excerpted from a total of over 49 referred papers)

- Wong, D.T., Nath, N., and Sninsky, J.J. (1985) "Identification of hepatitis B virus polypeptides encoded by the entire pre-s open reading frame." *J. Virol.* 55:223-231.
- Meyers, M.L., Vitvitskyi-Trepo, L., Nath, N., and Sninsky, J.J. (1986) "Hepatitis B virus polypeptide X: Expression in *E. coli* and identification of specific antibodies in sera from HBV-infected humans." *J. Virol.* 57:101-109.
- Schaeffer, E., Snyder, R., and Sninsky, J.J. (1986) "Identification and localization of pre-s-encoded polypeptides from woodchuck and ground squirrel hepatitis viruses." *J. Virol.* 57:173-182.
- Pugh, J.C., Sninsky, J.J., Summers, J.W., and Schaeffer, E. (1987) "Characterization of a pre-S polypeptide on the surfaces of infectious avian hepadnavirus particles." *J. Virol.* 61:1384-1390.
- Kwok, S., Mack, D.H., Mullis, K.B., Poiesz, B., Ehrlich, G., Blair, D., Friedman-Kien, A., and Sninsky, J.J. (1987) "Identification of human immunodeficiency virus sequences by using *in vitro* enzymatic amplification and oligomer cleavage detection." *J. Virol.* 61:1690-1694.

- Ou, C.-Y., Kwok, S., Mitchell, S.W., Mack, D. H., Sninsky, J.J., Krebs, J. W., Feorino, P., Warfield, D., and Schochetman, G. (1988) "DNA amplification for direct detection of HIV-I in DNA peripheral blood mononuclear cells." *Science* 239:295-297.
- Byrne, B., Li, J.J., Sninsky, J.J., and Poiesz, B. (1988) "Detection of HIV-1 RNA sequences by *in vitro* DNA amplification." *Nucl. Acids Res.* 16:4165.
- Bhagavati, S., Kula, R., Ehrlich, G., Kwok, S., Sninsky, J.J., Udami, V., and Poiesz, B. (1988) "Detection of human T cell lymphoma/leukemia virus-type 1 (HTLV-I) in the spinal fluid and blood of cases of chronic progressive myelopathy and clinical, radiological and electrophysiological profile of HTLV-I associated myelopathy." *N. Eng. Jour. Med.* 318:1141-1147.
- Mack, D. H., and Sninsky J.J. (1988) "A sensitive method for the identification of uncharacterized members of known virus groups: Hepadnavirus model system." *Proc. Natl. Acad. Sci. USA* 85:6977-6981.
- Kwok, S., Ehrlich, G., Poiesz, B., Kalish, R., and Sninsky J. J. (1988) "Enzymatic amplification of HTLV-I viral sequences from peripheral blood mononuclear cells and infected tissues." *Blood* 72:1117-1123.
- Mack, D., Bloch, W. Nath, N., and Sninsky, J.J. (1988) "Hepatitis B virus particles contain a polypeptide encoded by the largest open reading frame: A putative reverse transcriptase." *J. Virol.* 62:4786-4790.
- Kwok, S., Kellogg, D., Ehrlich, G., Poiesz, B., Bhagavati, S., and Sninsky, J.J. (1988) "Characterization of a sequence of human T cell leukemia virus type I from a patient with chronic progressive myelopathy." *J. Infec. Dis.* 158:1193-1197.
- Meyerhans, A., Cheynier, R., Albert, J., Seth, M., Kwok, S., Sninsky, J.J., Morfeldt-Manson, L., Asjö, B., and Wain-Hobson, S. (1989) "Temporal fluctuations in HIV quasispecies *in vivo* are not reflected by sequential HIV isolations." *Cell* 58:901-910.
- Shibata, D., Byrnes, R.K., Nathwani, B., Kwok, S., Sninsky, J.J., and Arnheim, N. (1989) "Human immunodeficiency viral DNA is readily found in lymph node biopsies from seropositive individuals. Analysis of fixed tissue using the polymerase chain reaction." *Am. J. Pathol.* 135:697-702.
- Kwok, S., Kellogg, D., Levenson, C., Spasic, D., Goda, L., and Sninsky, J.J. (1990) "Effects of primer-template mismatches on the polymerase chain reaction: Human immunodeficiency virus type 1 model studies." *Nucl. Acids Res.* 18:999-1005.
- Kwok, S., Gallo, D., Hanson, C.V., McKinney, N., Poiesz, B., and Sninsky, J.J. (1990) "High prevalence of HTLV-II among intravenous drug abusers: PCR confirmation and typing." *AIDS & Human Retroviruses* 6:559-563.
- Kellogg, D.E., Sninsky, J. and Kwok, S. (1990) "Quantitation of HIV-1 Proviral DNA Relative to Cellular DNA by the Polymerase Chain Reaction." *Analytical Biochemistry* 189:202-208.
- Ehrlich, H., Gelfand, D. and Sninsky, J. (1991) "Recent Advances in the Polymerase Chain Reaction". *Science* 255:1643-1651.
- Busch, M.P., Eble, B.E., Khayam-Bashi, H., Heilbron, D., Murphy, E.L., Kwok, S, Sninsky, J.J., Perkins, H.A., Vyas, G.N. (1991) "Evaluation of Screened Blood Donations for Human Immunodeficiency Virus Type 1 Infection by Culture and DNA Amplification of Pooled Cells". *The New Eng. Jour. of Med.* 325:1-5.
- Tada, M., Omata, M., Kawai, S., Saisho, H., Ohto, M., Saiki, R., and Sninsky, J.J. (1993) "Detection of *ras* Gene Mutation in Pancreatic Juice and Peripheral Blood of Patients with Pancreatic Adenocarcinoma". *Cancer Research* 53:2472-2474.
- Mulder, J., McKinney, N., Christopherson, C., Sninsky, J., Greenfield, L. and Kwok, S. (1993) "A Rapid and Simple PCR Assay for Quantitation of HIV RNA: Application to Acute Retroviral Infection" *Jour. of Clin. of Micro.* (in press).



**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME Mark E. SOBEL	POSITION TITLE Chief, Molecular Pathology Section		
EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Brandeis University, Waltham, MA	B.A.	1970	Psychology
Mount Sinai School of Medicine, New York	M.D.	1975	Medicine
Graduate School of the City University of New York	Ph.D.	1975	Biomedical Sciences
Children's Hospital Medical Center, Boston, MA	Resident	75-76	Pediatrics

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

1975-1976 Children's Hospital Medical Center (Boston), Intern in Medicine  
 1975-1976 Harvard University, Clinical Fellow in Pediatrics  
 1976-1981 National Cancer Institute, Postdoctoral Fellow; I. Pastan  
 1979-1980 Max Planck Institute for Biochemistry (Martinsried bei Munchen, Germany), Visiting Scientist  
 1981-1983 National Institute for Dental Research, Research Associate  
 1983-1992 National Cancer Institute, Senior Research Investigator, Laboratory of Pathology  
 1992-present National Cancer Institute, Chief, Molecular Pathology Section  
 1987-present American Society for Investigative Pathology, Director, Concepts in Molecular Biology Course  
 1989-present College of American Pathologists, Consultant, Molecular Pathology Resource Committee

*Honors and Awards:*

Phi Beta Kappa, 1969  
 John Leslie Award, Brandeis University, 1969  
 Michael Bokar Award, Brandeis University, 1970  
 Summa cum laude, Brandeis University, 1970  
 New York Diabetes Association Fellowship, 1971  
 Mount Sinai Graduate School of Biological Sciences Fellowship, 1973-1974  
 Basic Sciences Achievement Award, Mount Sinai School of Medicine, 1975  
 Arthritis Foundation Study Section, 1981-1984  
 Editorial Board, Calcified Tissue International, 1986-1991  
 Member, Minority Recruitment Networking Task Force, National Cancer Institute, 1987  
 Member, Ad hoc, NIH Pathobiochemistry Study Section, 1988  
 PHS Commendation Medal, 1989  
 Member, Research Grant Review Committee, American Cancer Society, 1991-present  
 Saul J. Horowitz, Jr. Memorial Award, Mount Sinai School of Medicine, 1991

*Representative Publications:* (excerpted from a total of over 70 refereed papers)

Steeg, P.S., Bevilacqua, G., Pozzati, R., Liotta, L.A., and Sobel, M.E. (1988) Expression of NM23, a gene associated with low tumor metastatic potential, is increased during adenovirus 2 E1a inhibition of experimental metastasis. *Cancer Res.* 48: 6550-6554.

- Castronovo, V., Taraboletti, G., Liotta, L.A., and Sobel, M.E. (1989) Modulation of laminin receptor expression by estrogen and progestins in human breast cancer cell lines. *J. Natl. Cancer Inst.* 81: 781-788, 1989.
- Sobel, M.E. (1990) Metastasis suppressor genes. *J. Natl. Cancer Inst.* 82: 267-276.
- Castronovo, V., Colin, C., Claysmith, A.P., Chen, P.H.S., Lifrange, E., Lambotte, R., Kruttsch, H.C., Liotta, L.A., and Sobel, M.E. (1990) Immunodetection of the metastasis-associated laminin receptor in human breast cancer cells obtained by fine needle aspiration biopsy. *Am. J. Pathol.* 137: 1373-1381.
- Cioce, V., Castronovo, V., Shmookler, B.M., Garbisa, S., Grigioni, W.F., Liotta, L.A., and Sobel, M.E. (1991) Increased expression of the laminin receptor in human colon cancer. *J. Natl. Cancer Inst.* 83: 29-36.
- Fernandez, M.-T., Castronovo, V., Rao, C.N., and Sobel, M.E. (1991) The high affinity murine laminin receptor is a member of a multicopy gene family. *Biochem. Biophys. Res. Commun.* 175: 84-90.
- Castronovo, V., Claysmith, A.P., Barker, K.T., Cioce, V., Kruttsch, H., and Sobel, M.E. (1991) Biosynthesis of the 67 kDa high affinity laminin receptor. *Biochem. Biophys. Res. Commun.* 177: 177-183.
- Castronovo, V., Taraboletti, G., and Sobel, M.E. (1991) Laminin receptor complementary DNA-deduced synthetic peptide inhibits cancer cell attachment to endothelium. *Cancer Res.* 51: 5672-5678.
- Castronovo, V., Taraboletti, G., and Sobel, M.E. (1991) Functional domains of the 67-kDa laminin receptor precursor. *J. Biol. Chem.* 266: 20440-20446.
- Demeter, L.M., Stoler, M.H., Sobel, M.E., Broker, T.R., and Chow, L.T. (1992) Expression of high affinity laminin receptor mRNA correlates with cell proliferation rather than invasion in human papillomavirus-associated cervical neoplasms. *Cancer Res.* 52: 1561-1567.
- Campo, E., Monteagudo, C., Castronovo, V., Claysmith, A.P., Fernandez, P.L., and Sobel, M.E. (1992) Detection of laminin receptor mRNA in human cancer cell lines and colorectal tissues by *in situ* hybridization. *Am. J. Pathol.* 141: 1073-1083.
- Castronovo, V., Campo, E., van den Brule, F., Claysmith, A.P., Cioce, V., Liu, F.-T., Fernandez, P.L., and Sobel, M.E. (1992) Inverse modulation of steady state mRNA levels of two non-integrin laminin binding proteins in human colon carcinoma. *J. Natl. Cancer Inst.* 84: 1161-1169.
- van den Brule, F., Engel, J., Stetler-Stevenson, W.G., Liu, F.-T., Sobel, M.E., and Castronovo, V. (1992) Genes involved in tumor invasion and metastasis are differentially modulated by estradiol and progestin in human breast cancer cells. *Int. J. Cancer* 52: 653-657.
- Taraboletti, G., Belotti, D., Giavazzi, R., Sobel, M.E., and Castronovo, V. (1993) Enhancement of metastatic potential of murine and human melanoma cells by laminin receptor peptide G: attachment of cancer cells to the subendothelial matrix as a pathway for hematogenous metastases. *J. Natl. Cancer Inst.* 82: 235-240.
- Sobel, M.E. (1993) Differential expression of the 67 kDa laminin receptor in cancer. *Sem. Cancer Biol.* 4: 311-317, 1993.
- Castronovo, V., Kusaka, M., Chariot, A., and Sobel, M.E. (1994) Homeobox genes: potential candidates for the transcriptional control of the transformed and invasive phenotype. *Biochem. Pharmacol.* in press.
- Bernstein, L.R., Ferris, D.K., Colburn, N.H., and Sobel, M.E. (1994) A family of MAP kinase related proteins interacts *in vivo* with the AP-1 transcription factor. *J. Biol. Chem.* in press.

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME <b>Steve S. SOMMER</b>		POSITION TITLE <b>Associate Professor of Molecular Biology</b>	
EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
University of Pennsylvania, Philadelphia, PA	B.A.	1972	Biophysics
Rockefeller University, New York, NY	Ph.D.	1978	Molecular Biology
Presbyterian Hospital, New York City, NY	M.D.	1979	Medicine

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

- 1979-1980 Resident, Surgical Pathology and Postmortem Section, Dr. Jose Costa, Chief; Laboratory of Pathology, Dr. Alan S. Rabson, Chief; National Cancer Institute, National Institutes of Health, Bethesda, MD
- 1980-1985 Medical Staff Fellow, Section of the Genetics of Simple Eukaryotes, Dr. Reed Wickner, Chief; Laboratory of Biochemical Pharmacology, Dr. Herbert Tabor, Chief; National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD. Research area: Molecular genetics of virus-like particles in yeast.
- 1982-1984 Multicenter Individual Fellowship in Clinical Genetics; sponsors, Dr. James Sidbury, Jr., Chief, Section on Developmental Biology and Human Nutrition, National Institute of Child Health and Human Development and Dr. Kenneth Rosenbaum, Director, Clinical Genetics, Children's Hospital National Medical Center, Washington, D.C.
- 7/85-7/90 Assistant Professor of Molecular Biology, Department of Biochemistry and Molecular Biology, Mayo Foundation School of Medicine, Rochester, MN.
- 7/90-present Associate Professor of Molecular Biology, Mayo Foundation, Rochester, MN

*Honors and Awards:*

- American Board of Medical Genetics Diplomate in Clinical Genetics, 1984  
American Board of Medical Genetics Diplomate in Clinical Molecular Genetics, 1993

*Representative Publications:* (excerpted from a total of 112 publications)*Development of Methodology for Information Retrieval from Nucleic Acids*

- Stoflet, E.S., Koeberl, D.D., Sarkar, G. and Sommer, S.S. (1988). Genomic amplification with transcript sequencing. *Science* 239:491-494.
- Sarkar, G., and Sommer, S.S. (1989). Access to a messenger RNA sequence or its protein product is not limited by tissue or species specificity. *Science* 244:331-334.
- Bottema, C.D.K., Koeberl, D.D., and Sommer, S.S. (1989). Direct carrier testing in 14 families with hemophilia B. *The Lancet* 2:526-529.
- Sarkar, G., and Sommer, S.S. (1991). Haplotyping by double PCR amplification of specific alleles. *BioTechniques* 10:436-440.

Sarkar, S., Yoon, H-S., and Sommer, S.S. (1992). Screening for mutations by RNA single-strand conformation polymorphism (rSSCP): Comparison with DNA-SSCP. *Nucleic Acids Res.* 20:871-878.

Sarkar, G., Yoon, H-S., and Sommer, S.S. (1992). Dideoxy fingerprinting (ddF): a rapid and efficient screen for the presence of mutations. *Genomics* 13:441-443.

#### *Germline Mutations in Humans*

Koeberl, D.D., Bottema, C.D.K., Buerstedde, J-M., and Sommer, S.S. (1989). Functionally important regions of the factor IX gene have a low rate of polymorphism and CpG is a dramatic hotspot of germline mutation. *Am. J. Hum. Genet.* 45:448-457.

Koeberl, D.D., Bottema, C.D.K., Ketterling, R.P., Bridges, P.J., Lillicrap, D.P., and Sommer, S.S. (1990). Mutations causing hemophilia B: direct estimate of the underlying rates of spontaneous germline transitions, transversions, and deletions in a human gene. *Am. J. Hum. Genet.* 47:202-217.

Ketterling, R.P., Bottema, C.D.K., Phillips, J.P., III, and Sommer, S.S. (1991). Evidence that descendants of three founders comprise about 25% of hemophilia in the United States. *Genomics* 10:1093-1096.

Ketterling, R.P., Vielhaber, E., Bottema, C.D.K., Schaid, D.J., Phillips, J.A., III, Sexauer, C., Kim, H., Gruppo, R., and Sommer, S.S. (1993). Germline origins of mutation in families with hemophilia B: the sex ratio varies with the type of mutation. *Am. J. Hum. Genet.* 52:152-166.

Gostout, B., Vielhaber, E., Ketterling, R.P., Yoon, H-S., Bottema, C.D.K., Kasper, C.K., Koerper, M., and Sommer, S.S. (1993). Germline mutations in the factor IX gene: a comparison of the pattern in Caucasians and non-Caucasians. *Hum. Molec. Genet.* 2:293-298.

Sommer, S.S. and Ketterling, R.P. (1993). A postulated mechanism for deletions with inversions. *Am. J. Hum. Genet.* 52:1016-1018.

Jacobson, D.P., Schmeling, P., and Sommer, S.S. (1993). Characterization of the patterns of polymorphism in a "cryptic repeat" reveals a novel type of hypervariable sequence. *Am. J. Hum. Genet.* 53:443-450.

Ketterling, R.P., Vielhaber, E.L., Lind, T.J., Thorland, E.C., and Sommer, S.S. (1994). The rates and patterns of deletions in a human gene in which deletions are uncommon. *Am. J. Hum. Genet.* In press.

Sommer, S.S. (1994). Does cancer kill the individual and save the species? *Human Mutation.* In press.

#### *Somatic Mutations in Breast Cancer*

Kovach, J.S., McGovern, R.M., Cassady, J.D., Swanson, S.K., Wold, L.E., Vogelstein, B., and Sommer, S.S. (1991). Direct sequencing from touch preparations of human carcinomas: analysis of p53 mutations in breast carcinomas. *J. Natl. Cancer Inst.* 83:1004-1009.

Sommer, S.S., Cunningham, J., McGovern, R.M., Saitoh, S., Schroeder, J.J., Wold, L.E., Kovach, J.S. (1992). Pattern of p53 gene mutations in breast cancers of women of the Midwestern United States. *J. Natl. Cancer Inst.* 84:246-252.

Blaszyk, H., Hartmann, A., Wold, L.E., Schroeder, J.J., McGovern, R.M., Sommer, S.S., and Kovach, J.S. (1994). A tandem CC-TT transition in the p53 gene of a breast cancer. *Human Molecular Genetics*, submitted.

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME Thierry SOUSSI	POSITION TITLE Professor		
EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
University Paris VII, Paris, France	Master	1978	Biochemistry
Institut for Cancer Research, Villejuif, France	Ph.D	1983	Virology
Institut for Cancer Research, Villejuif, France	Post Doc	1985	Molecular Biology
Ecole Normale Supérieure, Paris, France	Post Doc	1988	Molecular Biology

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

1985-1988 Institut for Cancer Research, **Post Doctoral Fellow**: P. May  
 1989-1991 Ecole Normale Supérieure, Paris, **Post Doctoral Fellow**; C. Jacq  
 1992-present Hôpital Saint Louis, Paris, **Head of Unit**  
 1983-1990 University P. & M. Curie; **Assistant Professor**  
 1991-present University P. & M. Curie; **Professor**

*Honors and Awards:*

National Cancer Foundation Award 1991  
 J.S. Bourgain Cancer Prize Award 1992

*Representative Publications:* (19 out of 32 papers)

- Soussi, T. (1986). DNA binding properties of the major structural protein of Simian Virus 40. *J. Virol.*, 59:740-742.
- Soussi, T., Caron de Fromentel, C., Méchali, M., May, P., and Kress, M. (1987). Cloning and characterization of a cDNA from *Xenopus laevis* coding for a protein homologous to human and murine p53. *Oncogene*, 1:71-78.
- Soussi, T., Caron de Fromentel, C., Sturzbecher, H.W., Ullrich, S., Jenkins, J., and May, P. (1989). Evolutionary conservation of the biochemical properties of p53 : Specific interaction of *Xenopus laevis* p53 with SV40 large T antigen and mammalian heat shock proteins 70. *J. of Virology*, 63:3894-3901.
- Gusse, M., Ghisdael, J., Evan, G., Soussi, T., and Méchali, M. (1989). Nuclear translocation of maternal cytoplasmic myc protein during early embryonic development. *Mol. Cell Biol.*, 9:5395-5403.
- Soussi, T., Caron de Fromentel, C., and May, P. (1990). Structural aspects of the p53 protein in relation to gene evolution. *Oncogene*, 5:945-952.
- Soussi, T. and Jonveaux, P. (1991). p53 gene alterations in human hematological malignancies: a review. *Nouv Rev Fr Hematol.*, 33:477-480.
- Caron de Fromentel, C. and Soussi, T. (1992). The p53 tumor suppressor gene: a model for investigating human mutagenesis. *Genes, Chromosomes and Cancer*, 4:1-15.

- Legros, Y., Mc Intyre, P., and Soussi, T. (1992). Cloning and biochemical characterization of hamster p53 *Genes*, 112:247-250.
- Caron de Fromentel, C., Padkel, C., Chapus, A., Baney, C., May, P., and Soussi, T. (1992). Rainbow trout p53: cDNA cloning and biochemical characterization. *Genes*, 112:241-245.
- Lidereau, R. and Soussi, T. (1992). Absence of p53 germ-line mutations in bilateral breast cancer patients *Human genetics* 89:250-252.
- Schlichtholz, B., Legros, Y., Gillet, D., Gaillard, C., Marty, M., Lane, D., Calvo, F., and Soussi, T. (1992). The immune response to p53 in breast cancer patients is directed against immunodominant epitopes unrelated to the mutational hot spot *Cancer Research*, 52:6380-6384.
- Legros, Y., Lacabanne, V., D'agay, M. F., Larsen, C. J., Pla, M., and Soussi, T. (1993). Production of human p53 specific monoclonal antibodies and their use in immunohistochemical studies of tumor cells *Bulletin Français du Cancer*, 10:102-110.
- Tchang, F., Gusse, M., Soussi, T., and Méchali, M. (1993). Stabilization and expression of high level of p53 during development of *Xenopus laevis* *Developmental Biology*, 159:163-172.
- Soussi T. (1993). p53 and cell transformation in *Advance in Cancer Biology*, In press.
- Legros, Y., Lafon, C., and Soussi, T. (1993). Presence of immunodominant antigenic sites in the amino- and carboxy-termini of human p53. *Oncogene*, In press.
- Lubin, R., Schlichtholz, B., Bengoufa, D., Zalcman, G., Trédaniel, J., Hirsch, A., Caron de Fromentel, C., Preudhomme, C., Fenaux, P., Fournier, G., Mangin, P., Laurent-Puig, P., Pelletier, G., Schlumberger, M., Desgrandchamps, F., Le Duc, A., Peyrat, J. P., Janin, N., Bressac, B., and Soussi, T. (1993). Analysis of p53 antibodies in patients with various cancer defines B-cell epitopes of human p53: Distribution on the primary structure and exposure on the protein surface *Cancer Research*, 53:5872-5876.
- Schlichtholz, B., Trédaniel, J., Lubin, R., Zalcman, G., Hirsch, A., and Soussi, T. (1994). Analysis of p53 antibodies in sera of patients with lung carcinoma define immunodominant regions in the p53 protein. *British J. Cancer*, In press.
- Hollstein M., Soussi, T. and Thomas, G. (1994). Medical diagnosis and p53 gene alteration: In press.
- Soussi, T., Legros, Y., Lubin, R., Ory, K., and Schlichtholz, B. (1994). Multifactorial analysis of p53 alteration in human cancer: a review. *Int. J. Cancer*, In press.

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME <b>Josef VÁGNER</b>		POSITION TITLE <b>Professor of Chemistry / Principal Investigator</b>	
EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Faculty of Org. Chem., Inst. of Technology, Prague	M.S.	1983	Chemistry,
Institute of Pharmacy and Biochemistry, Prague	Ph.D.	1990	Biochemistry
University of Minnesota, Dept. of Chem.	Postdoc	1992-present	Peptide Synthesis

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

Research Worker, Institute of Organic Chemistry and Biochemistry, Prague, 1983-1986.  
 Research Worker, Léčiva Pharmaceuticals, 1986-1990.  
 Scientific Worker, Institute of Sera and Vaccines, Prague, 1990-1992.  
 University of Minnesota, Department of Chemistry, Postdoctoral Fellow, 1992-present

*Research Interests and Experience:*

solid-phase and solution peptide synthesis, immunology, protein chemistry

*Relevant Professional Activities:*

Scientific Consultant, Institute of Feed Supplements and Veterinary Drugs, Jilové, 1990-1992.

*Representative Publications:* (excerpted from total of about 30 refereed papers)

- V. Krchňák, J. Vágner, M. Flegel, and O. Mach. Continuous-flow solid-phase peptide synthesis. *Tetrahedron Lett.* **28**, 4469-4472 (1987).
- V. Krchňák, J. Vágner, and M. Lebl. Noninvasive continuous monitoring of solid-phase peptide synthesis by acid-base indicator. *Int. J. Peptide Protein Res.* **32**, 415-416 (1988).
- V. Krchňák, J. Vágner, and M. Lebl. Amino acids and peptides. Part CCVI. Noninvasive continuous monitoring of solid-phase peptide synthesis by acid-base indicator. *Coll. Czech. Chem. Commun.* **53**, 2542-2548 (1988).
- V. Krchňák, J. Vágner, and I. Hirsh. Simultaneous synthesis of sequence unrelated peptides derived from proteins of human papillomaviruses. *Coll. Czech. Chem. Commun.* **53**, 2645-2653 (1988).
- V. Krchňák, J. Vágner, and O. Mach. Multiple continuous-flow solid-phase peptide synthesis. The synthesis of an HIV antigenic peptide and its omission analogues. *Int. J. Peptide Protein Res.* **33**, 209-213 (1989).
- T. Jandlová, J. Bubeník, V. Krchňák, J. Vágner, N.N. Voitenok, E. Gren, J. Šimová, and O. Mach. Solid phase enzyme-immunoassay of human interleukin-2 utilizing antibodies against synthetic IL-2 peptides. *Folia Biologica* **35**, 205-217 (1989).

- A.L. Luzzati, O. Pugliese, E. Giacomini, L. Giordani, F. Quintieri, T. Hraba, O. Mach, V. Krchňák, and J. Vágner. Immunoregulatory effect of a synthetic peptide corresponding to a region of protein p24 of HIV. *Folia Biologica* **36**, 71-77 (1990).
- V. Krchňák, J. Vágner, J. Novák, A. Suchánková, and J. Roubal. A general procedure for evaluation of immunological relevance of sythetic peptides: Peptides synthesized on paper in enzyme-linked immunosorbent assay. *Anal. Biochem.* **189**, 80-83 (1990).
- V. Krchňák and J. Vágner. Color-monitored solid-phase multiple peptide synthesis under low-pressure continuous-flow conditions. *Peptide Res.* **3**, 182-193 (1990).
- V. Krchňák, J. Vágner, A. Suchánková, M. Krčmář, L. Ritterová, L., and V. Vonka. Synthetic peptide derived from E7 region of human papillomavirus type 16 used as antigens in ELISA. *J. Gen. Virol.* **71**, 2719-2724 (1990).
- J. Vágner, P. Kocna, and V. Krchňák. Continuous-flow synthesis of a-gliadin peptides in an ultrasonic field and assay of their inhibition of intestinal sucrase activity. *Peptide Res.* **4**, 284-288 (1991).
- A. Suchánková, L. Ritterová, M. Krčmář, V. Krchňák, J. Vágner, I. Jochmus, L. Gissmann, J. Kaňka, and V. Vonka. Comparison of ELISA and Western blotting for human papillomavirus type 16 E7 antibody determination. *J. Gen. Virol.* **72**, 2577-2581 (1991).
- A. Suchánková, V. Krchňák, J. Vágner, M. Krčmář, L. Ritterová, and V. Vonka. Epitope mapping of the human papillomavirus type 16-E4 protein by means of synthetic peptides. *J. Gen. Virol.* **73**, 429-432 (1992).
- V. Krchňák and J. Vágner. Prediction and handling of difficult sequences in solid-phase peptide synthesis. In "Innovation and Perspectives in Solid Phase Synthesis and Related Technologies: Peptides, Polypeptides and Oligonucleotides 1992" (R. Epton, ed.), Intercept, Andover, England, 1992, pp. 419-424.
- V. Krchňák, J. Vágner, L. Ráček, and J. Novák. Epitope mapping using peptide synthesized on paper: gag proteins of HIV. In "25 Years of Immunoenzymatic Techniques," Athens, 1992, in press.
- J. Vágner, V. Krchňák, J. Pícha, D. Píchová, and M. Fusek. Colour-monitored solid-phase multiple peptide synthesis under low-pressure continuous-flow conditions. Synthesis of medium-size peptides: the propart of human procathepsin-D and the growth-hormone releasing factor. *Coll. Czech. Chem. Commun.*, **58**, 435-444 (1993).
- Z. Flegelová, J. Vágner, and V. Krchňák. An alternative approach to sulphated peptides: Caerulein. In "Peptides 1992: Proceedings of the Twenty-Second European Peptide Symposium" (C.H. Schneider and A.N. Eberle, eds.), Escom Science Publishers, Leiden, The Netherlands, 1993, pp. 351-352.
- V. Krchňák, Z. Flegelová, and J. Vágner. Aggregation of peptidyl-resin in solid-phase peptide synthesis. Prediction of difficult sequences. *Int. J. Peptide Prot. Res.* **42**, 450-454 (1993).
- A. Suchánková, M. Krčmář, V. Krchňák, J. Vágner, and V. Vonka. Range of HPV 16 E7 antibodies in cervical cancer patients and healthy subjects. *Int. J. Cancer*, in press (1993).
- J. Vágner, V. Krchňák, N.F. Sepetov, P. Štrop, K.S. Lam, G. Barany, and M. Lebl. Novel Methodology for Differentiation of "Surface" and "Interior" Areas of Polyoxyethylene-Polystyrene (POE-PS) Supports: Application to "Library" Screening Procedures. In "Innovation and Perspectives in Solid Phase Synthesis and Related Technologies" (R. Epton, ed.), SPCC (UK) Ltd., Birmingham, in press (1994).



**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME <b>Guangyi WANG</b>	POSITION TITLE <b>Postdoctorate</b>		
EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Beijing Institute of Technology	B.S.	1976	Organic Synthesis
Beijing Institute of Technology	M.S.	1982	Organic Synthesis
University of Basel, Basel, Switzerland	Ph.D.	1987	Organic Chemistry

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

Shenyang Institute of Chemical Technology, 1982-1985, Lecturer of Organic Chemistry, Department of Applied Chemistry

University of Arizona, 1987-1988, Postdoctoral Research Associate, Department of Chemistry

University of Maryland, 1988-1989, Postdoctoral Research Associate, Department of Chemistry

Purdue University, 1989-present, Postdoctoral Research Associate, Department of Medicinal Chemistry and Pharmacognosy

*Representative Publications:*

"Synthesis of Aromatic Trinitromethyl Compounds," *Zhongguo Binggong Xuebao*, **1983**, 2, 1.

"Charge Dispersal in 1- and 2-Adamantyl Cations," *Tetrahedron Letters*, **1987**, 28 (12), 1247.

"Inductivity and Bridging in Ammonium and Carbenium Ions," *Angew. Chem. Int. Ed.*, **1988**, 27, 714.

"Bridging Strain in Norbornyl and Oxanorbornyl Cations," *Helv. Chim. Acta*, **1988**, 71, 1017.

"Solvolytic Cyclization of 4,15-Anhydroverrucarol. A Facile Trichothecene-10,13-Cyclotrichothecene Rearrangement," *J. Org. Chem.*, **1989**, 54, 4493.

"Synthesis and Stereochemistry of 4-Substituted 2-Azaadamantanols," *Huaxue Xuebao*, **1990**, 48 (4), 482.

"A Bond-Forming Initiation Mechanism for Spontaneous Polymerisations Accompanying Diels-Alder Reactions," *J. Chem. Soc., Chem. Commun.*, **1991**, 18, 1280.

"A Convenient Method for the Conversion of  $\alpha$ -Tetralones to Aryl Acetates," *Syn. Commun.*, **1991**, 21, 989.

"C-5 Substituted Nucleoside Analogs" *Synthetic Letters*. **1992**, 179-188.

"Controlled Oxidation of Dimethyl Derivatives of Pyridine, 2,2'-Bipyridine, and 1,10-Phenanthroline," *Synthetic Letters* **1992**, 422-425.

"Synthesis of Oligonucleotides Containing N<sup>2</sup>-[2(imidazole-4-acetamido)ethyl]-2'-deoxyguanosine," *Tetrahedron Letts.* **1993**, 34, 6725-6728.

"Synthesis of Oligonucleotides Containing N<sup>2</sup>-(5-Carboxypentyl)-2'-deoxyguanosine and 5'-[2-(4'-Methyl-2,2'-dipyridyl-4-carboxamido)ethylthio]-2'-deoxyuridine," *Tetrahedron Letts.* **1993**, 34, 6721-6724.

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME Perrin C. WHITE		POSITION TITLE Professor of Pediatrics	
EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Harvard College, Cambridge, MA	B.A.	1972	Biochemistry
Harvard Medical School, Boston, MA	M.D.	1976	Medicine

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

1976-1978 Intern to Assistant Resident, Dept. of Pediatrics, Johns Hopkins Hospital, Baltimore, MD  
 1978-1980 Postdoctoral Fellow, Developmental & Molecular Biology, Rockefeller University, NY  
 1980-1981 Assistant Pediatrician, New York Hospital, New York, NY  
 1980-1981 Guest Investigator, Rockefeller University, New York, NY  
 1981-1985 Research Associate, Sloan-Kettering Institute, New York, NY  
 1981-1987 Assistant Professor of Pediatrics, Cornell University Medical College, New York, NY  
 1985-1990 Adjunct Assistant to Associate Member, Sloan-Kettering Institute, New York, NY  
 1985-present Director, Laboratory of Molecular Endocrinology, Cornell University Medical College, NY  
 1987-1991 Associate Professor of Pediatrics, Cornell University Medical College, New York, NY  
 1991-present Professor of Pediatrics, Cornell University Medical College, New York, NY

*Honors and Awards:*

Westinghouse Scholar	1968-1972
National Merit Scholar	1968-1972
High Honors in Biochemical Sciences, Harvard College	1972
American Cancer Society Fellow	1978-1980
Norman and Rosita Winston Fellow in Biomedical Research	1982-1984
Young Investigator Award, American Society for Histocompatibility and Immunogenetics	1984
Andrew J. Mellon Teacher-Scientist	1985-1987
Irma T. Hirschl Trust Scholar	1989-present
Ernst Oppenheimer Award, Endocrine Society	1991

*Representative Publications:*

White PC, Vitek A, Dupont B, New MI. (1988) Characterization of frequent deletions causing steroid 21-hydroxylase deficiency. *Proc. Natl. Acad. Sci. USA* 85:4436-4440.

Agarwal AK, Monder C, Eckstein B, White PC. (1989) Cloning and expression of rat cDNA encoding corticosteroid 11 $\alpha$ -dehydrogenase. *J. Biol. Chem.* 264:18939-18943.

Mornet E, Dupont J, Vitek A, White PC. (1989) Characterization of two genes encoding human steroid 11 $\alpha$ -hydroxylase (P450c11). *J. Biol. Chem.* 264:20961-20967.

Speiser PW, Laforgia N, Kato K, Pareira J, Khan R, Yang SY, Whorwood C, White PC, et al. (1990) First trimester prenatal treatment and molecular genetic diagnosis of congenital adrenal hyperplasia (21-hydroxylase deficiency). *J. Clin. Endocrinol. Metab.* 70:838-848.

- Tusie-Luna MT, Traktman P, White PC. (1990) Determination of functional effects of mutations in the steroid 21-hydroxylase gene (CYP21) using recombinant vaccinia virus. *J. Biol. Chem.* 265:20916-20922.
- Mornet E, Crete P, Kuttann F, Raux-Demay MC, Boue J, White PC, Boue A. (1991) Distribution of deletions and seven point mutations on CYP21B genes in three clinical forms of steroid 21-hydroxylase deficiency. *Am. J. Hum. Genet.* 48:79-88.
- Speiser PW, Agdere L, Ueshiba H, White PC, New MI. (1991) Aldosterone synthesis in salt-wasting congenital adrenal hyperplasia with complete absence of adrenal 21-hydroxylase. *N. Engl. J. Med.* 324: 145-149.
- White PC, Dupont J, New MI, Leiberman E, Hochberg Z, Rosler A. (1991) A mutation in CYP11B1 (Arg-448 to His) associated with steroid 11-hydroxylase deficiency in Jews of Moroccan origin. *J. Clin. Invest.* 87:1664-1667.
- Tusie-Luna MT, Speiser PW, Dumic M, New MI, White PC. (1991) A mutation (Pro-30 to Leu) in CYP21 represents a potential nonclassic steroid 21-hydroxylase deficiency allele. *Mol. Endocrinol.* 5: 685-692.
- Tannin GM, Agarwal AK, Monder C, New MI, White PC. (1991) The human gene for 11-hydroxysteroid dehydrogenase: structure, tissue distribution and chromosomal localization. *J. Biol. Chem.* 266:16553-16558.
- Speiser PW, New MI, Tannin GM, Pickering D, Yang SY, White PC. (1992) Genotype of Yupik Eskimos with congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *Human. Genetics.* 88:647-648.
- White PC, Vitek J, Lahita RG, Speiser PW. (1992) Polymorphism in the RD (D6S45) gene. *Human. Genetics.* 89:243-244.
- Pascoe L, Curnow KM, Slutsker L, Rosler A, White PC. (1992) Mutations in the human CYP11B2 (aldosterone synthase) gene causing corticosterone methyloxidase II deficiency. *Proc. Natl. Acad. Sci. USA* 89:4996-5000.
- Speiser PW, Dupont J, Zhu D, Serrat G, Buegeleisen M, Tusie-Luna MT, Lesser M, New MI, White PC. (1992) Disease expression and molecular genotype in congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *J. Clin. Invest.* 90:584-595.
- Curnow KM, Pascoe L, White PC. (1992) Genetic analysis of the human type-1 angiotensin II receptor. *Mol. Endocrinol.* 6:1113-1118.
- Helmberg A, Tusie-Luna MT, Tabarelli M, Kofler R, White PC. (1992) R339H and P453S: CYP21 mutations associated with nonclassic steroid 21-hydroxylase deficiency that are not apparent gene conversions. *Mol. Endocrinol.* 6:1318-1322.
- Pascoe L, Curnow KM, Slutsker L, Connell J, Speiser PW, New, MI, White PC. (1992) Glucocorticoid suppressible hyperaldosteronism results from hybrid genes created by unequal crossovers between CYP11B1 and CYP11B2. *Proc. Natl. Acad. Sci. USA* 89:8327-8331.
- Nikkilä H, Tannin GM, New MI, Taylor NF, Kalaitzoglou G, Monder C, White PC. (1993) Defects in the HSD11 gene encoding 11-hydroxysteroid dehydrogenase are not found in patients with apparent mineralocorticoid excess or 11-oxoreductase deficiency. *J. Clin. Endocrinol. Metab.* in press.
- Curnow KM, Slutsker L, Vitek J, Cole T, Speiser PW, New MI, White PC, Pascoe L. (1993) Mutations in the CYP11B1 gene causing congenital adrenal hyperplasia and hypertension cluster in exons 6,7 and 8. *Proc. Natl. Acad. Sci. USA* 90:4552-4556.

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME Geoffrey G. WILSON	POSITION TITLE Group leader / Industrial collaborator
----------------------------	--

EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
University of Sussex, UK	B.Sc.	1971	Biology
University of Sussex, UK	Ph.D.	1976	Microbial Genetics

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

1976-1977 University of Edinburgh, Molecular Biology; Postdoctoral Fellow; N. Murray  
 1977-1978 University of California Berkeley, Molecular Biology; Postdoctoral Fellow; H. Echols  
 1978-1980 Yale University Medical School, Biochemistry; Postdoctoral Fellow; W. Konigsberg  
 1980-present New England Biolabs, Inc., Beverly, MA; Research group leader

*Representative Publications:*

- Howard, K.A., Card, C., Benner, J.S., Callahan, H.L., Maunus, R., Silber, K., Wilson, G. and Brooks, J.E. (1986). Cloning the *DdeI* restriction-modification system using a two-step method. *Nucleic Acids Res.* 14:7939-7951.
- Raleigh, E.A. and Wilson, G. (1986). *Escherichia coli* K-12 restricts DNA containing 5-methylcytosine. *Proc. Natl. Acad. Sci. USA* 83:9070-9074.
- Caserta, M., Zacharias, W., Nwankwo, D., Wilson, G.G. and Wells, R.D. (1987). Cloning, sequencing, in vivo promoter mapping, and expression in *Escherichia coli* of the gene for the *HhaI* methyltransferase. *J. Biol. Chem.* 262:4770-4777.
- Ehrlich, M., Wilson, G.G., Kenneth, C.K. and Gehrke, C.W. (1987). N4-Methylcytosine as a minor base in bacterial DNA. *J. Bacteriol.* 169:939-943.
- Nwankwo, D. and Wilson, G. (1987). Cloning of two type II methylase genes that recognise asymmetric nucleotide sequences: *FokI* and *HgaI*. *Mol. Gen. Genet.* 209:570-574.
- Slatko, B.E., Benner, J.S., Jager-Quinton, T., Moran, L.S., Simcox, T.G., VanCott, E.M. and Wilson, G.G. (1987). Cloning, sequencing and expression of the *TaqI* restriction-modification system. *Nucleic Acids Res.* 15:9781-9796.
- Barsomian, J.M., Card, C.O. and Wilson, G.G. (1988). Cloning of the *HhaI* and *HinPI* restriction-modification systems. *Gene* 74:5-7.
- Chandrasegaran, S., Lunnen, K.D., Smith, H.O. and Wilson, G.G. (1988). Cloning and sequencing the *Hinfi* restriction and modification genes. *Gene* 70:387-392.
- Lunnen, K.D., Barsomian, J.M., Camp, R.R., Card, C.O., Chen, S.Z., Croft, R., Looney, M.C., Meda, M.M., Moran, L.S., Nwankwo, D.O., Slatko, B.E., Van Cott, E.M. and Wilson, G.G. (1988). Cloning Type II restriction and modification genes. *Gene* 74:25-32.

- Nwankwo, D.O. and Wilson, G.G. (1988). Cloning and expression of the *MspI* restriction and modification genes. *Gene* 64:1-8.
- Slatko, B.E., Croft, R., Moran, L.S. and Wilson, G.G. (1988). Cloning and analysis of the *HaeIII* and *HaeII* methyltransferase genes. *Gene* 74:45-50.
- Van Cott, E.M. and Wilson, G.G. (1988). Cloning the *FnuDI*, *NaeI*, *NcoI* and *XbaI* restriction-modification systems. *Gene* 74:55-59.
- Wilson, G.G. (1988). Type II restriction-modification systems. *Trends in Genetics* 4:314-318.
- Brooks, J.E., Benner, J.S., Heiter, D.F., Silber, K.R., Sznyter, L.A., Jager-Quinton, T., Moran, L.S., Slatko, B.E., Wilson, G.G. and Nwankwo, D.O. (1989). Cloning the *BamHI* restriction modification system. *Nucleic Acids Res.* 17:979-997.
- Landry, D., Looney, M.C., Feehery, G.R., Slatko, B.E., Jack, W.E., Schildkraut, I. and Wilson, G.G. (1989). *M.FokI* methylates adenine in both strands of its asymmetric recognition sequence. *Gene* 77:1-10.
- Looney, M.C., Moran, L.S., Jack, W.E., Feehery, G.R., Benner, J.S., Slatko, B.E. and Wilson, G.G. (1989). Nucleotide sequence of the *FokI* restriction-modification system: separate strand-specificity domains in the methyltransferase. *Gene* 80:193-208.
- Lunnen, K.D., Morgan, R.D., Timan, C.J., Krzycki, J.A., Reeve, J.N. and Wilson, G.G. (1989). Characterization and cloning of *MwoI* (GCN7GC), a new type-II restriction-modification system from *Methanobacterium wolfei*. *Gene* 77:11-19.
- Card, C.O., Wilson, G.G., Weule, K., Hasapes, J., Kiss, A. and Roberts, R.J. (1990). Cloning and characterization of the *HpaII* methylase gene. *Nucleic Acids Res.* 18:1377-1383.
- Renbaum, P., Abrahamove, D., Fainsod, A., Wilson, G.G., Rottem, S. and Razin, A. (1990). Cloning, characterization, and expression in *Escherichia coli* of the gene coding for the CpG DNA methylase from *Spiroplasma* sp. strain MQ1(M.SssI). *Nucleic Acids Res.* 18:1145-1152.
- Wilson, G.G. (1991). Organization of restriction-modification systems. *Nucleic Acids Res.* 19:2539-2566.
- Wilson, G.G. and Murray, N.E. (1991). Restriction and Modification Systems. *Annu. Rev. Genet.* 25:585-627.
- Barany, F., Slatko, B., Danzitz, M., Cowburn, D., Schildkraut, I. and Wilson, G.G. (1992). The corrected nucleotide sequences of the *TaqI* restriction and modification enzymes reveal a thirteen-codon overlap. *Gene* 112:91-95.
- Landry, D., Barsomian, J.M., Feehery, G.R. and Wilson, G.G. (1992). Characterization of Type II DNA-Methyltransferases. *Methods Enzymol.* 216:244-259.
- Wilson, G.G. (1992). Amino acid sequence arrangements of DNA-methyltransferases. *Methods Enzymol.* 216:259-279.
- Zhang, B., Tao, T., Wilson, G.G. and Blumenthal, R.M. (1993). The *M.AluI* DNA-(cytosine C5)-methyltransferase has an unusually large, partially dispensable, variable region. *Nucleic Acids Res.* 21:905-911.

**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME Vincent L. WILSON		POSITION TITLE Director / Associate Professor	
EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Sonoma State College, Rohnert Park, CA	B.S.	1973	Chemistry
University of California, Davis, CA	M.S.	1976	Physical Chemistry
Oregon State University, Corvallis, OR	Ph.D.	1980	Pharma. & Toxicology

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

**Professional Experience:**

9/72 - 7/73 Chemist, Central Pathology Lab, Inc., Santa Rosa, CA  
 4/78 - 8/80 Analytical Chemist, GS-7, Environmental Protection Agency, Corvallis Environmental Research Laboratory, Corvallis, OR  
 9/80 - 10/82 Postdoctoral Fellow (NIH Fellowship), USC Comprehensive Cancer Center, Children's Hospital, Los Angeles, CA  
 10/82 - 6/88 Senior Staff Fellow and Radiation Safety Officer, Laboratory of Human Carcinogenesis, National Cancer Institute, NIH, Bethesda, MD  
 9/86 - 10/86 Visiting Scientist, Danish Cancer Society, Lab of Environmental Carcinogenesis, Fibigerlaboratoriet, Copenhagen, Denmark  
 6/88 - Present Director, Laboratory of Molecular Genetics/Oncology, The Children's Hospital, Denver, CO  
 3/89 - Present Associate Professor, Department of Pathology, Univ. Colorado School of Medicine, Denver  
 10/90 - Present Assistant Radiation Protection Officer, The Children's Hospital, Denver, CO

**Honors and Awards:**

7/91 - Present Molecular Genetics Committee (Chairman, 7/91 - 8/93) of the Mountain States Regional Genetic Services Network, and Colorado representative on the Steering/Planning Committee of MSRGSN, and DNA Testing Subcommittee of CORN Quality Assurance Committee  
 1/91 - 1/95 Certified by the New York State Department of Public Health to Direct a clinical laboratory in Genetic Testing Services.

**Representative Publications:**

Wilson, V.L. and Jones, P.A. (1983) Inhibition of DNA methylation by chemical carcinogens *in vitro*. *Cell* 32: 239-246.  
 Wilson, V.L. and Jones, P.A. (1983) DNA methylation decreases in aging but not in immortal cells. *Science* 220: 1055-1057.  
 Wilson, V.L., Jones, P.A., & Momparler, R.L. (1983) Inhibition of DNA methylation in L1210 leukemic cells by 5-aza-2'-deoxycytidine as a possible component of chemotherapeutic action. *Cancer Res.* 43: 3493-3496.  
 Wilson, V.L., Smith, R.A., Autrup, H., Krokan, H., Musci, D.E., Le, N.-N.-T., Longoria, J., Ziska, D., & Harris, C.C. (1986) Genomic 5-methylcytosine determination by <sup>32</sup>P postlabeling analysis. *Anal. Biochem.* 152: 275-284.

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**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME Emily S. WINN-DEEN	POSITION TITLE Staff Scientist
----------------------------	-----------------------------------

EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Lehigh University, Bethlehem, PA	B.S.	1974	Chemistry
Boston University, Boston, MA	Ph.D.	1978	Chemistry

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

1989-present      Applied Biosystems, Staff Scientist, R&D  
 1988-1989      Ciba-Corning Diagnostics, Senior Biotechnology Development Scientist, R&D  
 1987-1988      SDI Diagnostics, Director R&D  
 1980-1987      Behring Diagnostics, Group Leader/Manager, R&D  
 1978-1980      Ames Division of Miles Laboratories, Research Scientist, R&D

*Professional Activities :*

Member, American Association for Clinical Chemistry (AACC), American Chemical Society (ACS),  
 American Society for Microbiology (ASM), American Society for Human Genetics (ASHG),  
 American Association for the Advancement of Science (AAAS)  
 Advisor, *Women in Science Program*, San Diego State University, 1982-1983  
 Advisor, *Educational Bridges to Options in High Technology Employment*, San Diego State University,  
 1984-1985  
 Symposium Chair, *Gene Probe Technology: From Theory to Practice*, San Diego Section of the AACC,  
 1986  
 Vice-Chair, San Diego Section of the AACC, 1987  
 Program Chair, *Gene Probe Technology II: The San Diego Conference*, 1987  
 Chair, San Diego Section of the AACC, 1988  
 Chair, Molecular Pathology Division, AACC, 1989-1992  
 Secretary, Northern California Section of the AACC, 1993  
 Member, NCCLS Subcommittee on Molecular Genetics, 1993-present

*Honors and Awards:*

Customer Acceptance Award, Behring Diagnostics, 1981  
 Behring Award, Behring Diagnostics, 1985  
 Harold Van Remortel Service Award, Northern California Section of the AACC, 1993

*Representative Publications:* (excerpted from a total of over 30 refereed papers)

Winn, E., Hu, S.P., Hochschwender, S. and Laursen, R. (1980). Studies on the Lysine Binding Site of Human Plasminogen, The Effect of Ligand Structure on the Binding of Lysine Analogs to Plasminogen. *Eur. J. Biochem.* **104**:579-586.

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**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME Peiming ZHANG	POSITION TITLE Postdoctorate		
EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
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Hunan Normal University, Changsha, Hunan, Prov.	B.S.	1982	Organic Synthesis
Beijing Institute of Chemistry, Academia Sinica	M.S.	1986	Organic Synthesis
Beijing Institute of Chemistry, Academia Sinica	Ph.D.	1990	Organic Chemistry

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

*Professional Experience:*

Purdue University, 1990-present, Postdoctoral Research Associate, Department of Medicinal Chemistry and Pharmacognosy

Walther Cancer Institute, 1993 - present, Senior Scientific Staff

*Representative Publications:*

- Z. Wang, P. Zhang, and S. Sun, "Synthesis and Characterization of Polymer Derivatives of Cis-platinum Complexes," *Chinese Journal of Polymer Science* **1987**, 4, 360.
- P. Zhang, F. Guo, and Z. Huang, "Synthesis of Triacetoxhydroxycalix(4)arene and Crystal Structure of Its Acetonitrile Clathrate," *Acta Chimica Sinica* **1989**, 47, 732.
- D. Bergstrom and P. Zhang, "An Efficient Route to C-4 Linked Imidazole Nucleosides: Synthesis of 2-Carbamoyl-4-(2'-deoxy-b-D-ribofuranosyl)imidazole," *Tetrahedron Letters* **1991**, 32, 6485.
- P. Zhang, F. Guo, and Z. Huang, "The Studies of the Inclusion Property of Tetrahydrocalix[4]arene," *Acta Chimica Sinica* **1992**, 50, 209.
- P. Zhang, et. al "Oligonucleotides Having Universal Nucleoside Spacers," patent application, submitted September 16, **1992**.
- P. Zhang, et. al "A Universal Nucleoside for Use at Ambiguous Sites in DNA Sequencing and PCR Primers," *Nature* (submitted).

# **NEW METHODS FOR CANCER DETECTION**

## **An Overview**

**Principal Investigator: Francis Barany  
Cornell University Medical College**

**Co-Investigator: Aneel Aggarwal  
College of Physicians & Surgeons  
of Columbia University**

**Co-Investigator: George Barany  
University of Minnesota**

**Co-Investigator: Donald Bergstrom  
Purdue University**

**Co-Investigator: Neil Hackett  
Cornell University Medical College**

**Co-Investigator: Robert P. Hammer  
Louisiana State University**

**Co-Investigator: Matthew Lubin  
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**Co-Investigator: Vincent Wilson  
The Childrens Hospital  
University of Colorado School of Medicine**

## A. GOALS:

The goal of this proposal is to develop three mutation detection technologies that will allow researchers and clinical oncologists to improve cancer care.

In the last decade many oncogenes and tumor suppressor genes have been described. Mutations in these growth-regulating genes are responsible for the development of cancers [1]. Understanding their functions in cancer biology has opened a new window into the mechanisms of tumorigenesis. A multi-step model for the development of colon cancer involving the adenomatous polyposis coli (APC) gene, the ras oncogene, the p53 gene and the "deleted in colon cancer" (DCC) gene is generally accepted (see Fig. 1) [2]. These genes have also been implicated in the development of tumors of the brain, breast, bladder, stomach, lung and liver [2-15]. This knowledge, however, has not significantly changed the care of patients with these cancers. Predicting the clinical and biological behavior of cancers from their genetic alterations has become a major goal of cancer genetics research.

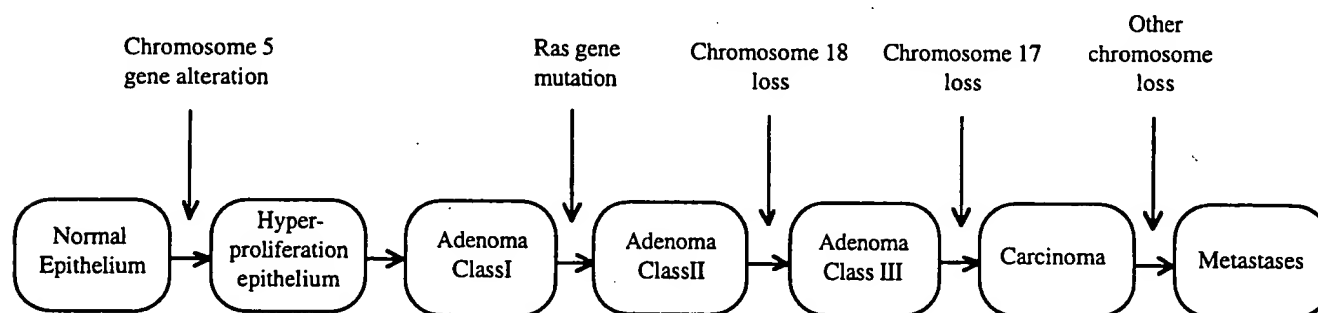


Fig. 1. The Vogelstein multi-step model for the development of colon cancers. Adapted from [2].

There are several lines of evidence supporting the hypothesis that tumor behavior can be predicted by genetic alterations. In childhood neuroblastomas N-myc gene amplification, and deletions of chromosome 1p correlate significantly with prognosis. (Reviewed in [16].) Hyper diploid or near triploid tumors are usually localized and typically have a good prognosis. Children whose tumors demonstrate a near-diploid chromosome complement generally have a slow, fatal progression. Neuroblastomas that are tetraploid or near-diploid with deletions of chromosome 1p, or N-myc gene amplification are rapidly fatal.

Indirect evidence for the effects of specific mutations (allele-specific effects) is beginning to accumulate in inherited conditions. For example, Familial Polyposis Coli (FPC) is an inherited, colon-cancer predisposing condition caused by mutations in the APC gene [17, 18]. Considerable variability of expression exists in FPC. Among the extracolonic effects seen in this condition is congenital hypertrophy of the retinal pigment epithelium, which is rarely seen in patients whose mutations occur before exon 9, and almost invariably present in patients whose mutations occur after this exon [19]. More recently the germline mutations in the *RET* oncogene have been shown to result in any of four phenotypes: familial medullary thyroid carcinoma (FMTC); multiple endocrine neoplasia, type 2A, (MEN2A); multiple endocrine neoplasia, type 2B, (MEN2B); and Hirschprung disease, a non-cancerous condition affecting the motility of the colon [20-22]. The phenotype seems to depend on the type of mutation and its location in the gene. Hirschprung disease cases are associated with deletions.

The clinical significance of genetic alterations in adult tumors has been extensively studied in the case of breast cancer. Tumor suppressor gene mutations and oncogene expression have been investigated in breast cancers as prognostic indicators, especially in "lymph node-negative" cases. (Reviewed in [15]). Overexpression of p53 measured by immunohistochemical staining correlates with earlier relapse and shortened survival [23-25]. Breast cancer p53 gene mutations are similarly associated with poor outcome [26-

28]. However, DNA analysis may be more informative than immunohistochemical analysis, since different mutations result in proteins with different functions [29-31]. For example, p53 proteins with mutations in exons 5 and 6 are capable of binding heat shock protein (hsp) 70 and are immunogenic. Mutations in exons 7 and 8 do not allow p53 to bind hsp 70 [30].

In 1990 an NIH Consensus Conference on the treatment of early stage breast cancer identified, as an area for future research, the development of risk factor profiles, "to allow identification of such groups that (a) may be treated with surgical excision with irradiation, (b) do not require axillary node dissection, and do not require systemic therapy" [32]. Studies of amplification and overexpression of oncogenes in breast cancers have focused on the HER2/neu, c-myc and int-2 genes. HER-2/neu gene amplification correlates with mRNA and protein overexpression [33]. HER-2/neu amplification and overexpression may be useful indicators of prognosis in breast cancer [34-37]. Overexpression of this gene and the *ras* gene product may act in synergy, producing a poor clinical outcome [38]. Similarly, amplification in the c-myc gene has been correlated with poor prognosis in breast cancer [39, 40] but other studies have not found the same result [41, 42]. Amplification of the int-2 gene has also been associated with large tumor size, reduced time to relapse and shorter survival in breast cancer cases [41].

While these studies often show statistical significance between breast cancer prognosis and the gene amplification or its expression, there are conflicting results and their clinical utility generally does not surpass other prognostic factors. The full nature of carcinogenesis in adult-onset tumors is likely to be extremely complex. To understand the genetic progression and different subsets of specific cancers, clinical and basic scientists will have to continue to make correlations between the biological characteristics of tumors and their clinical outcomes. To succeed in this, researchers will have to be able to investigate more genes from smaller amounts of tumor tissue. DNA technology holds the greatest promise for unlocking the vast amount of information stored in cancers, because the genetic code can be amplified without loss of information.

Large scale studies correlating genetic alterations with disease outcomes will be necessary for defining the clinical effects of various mutation combinations. Accomplishing this will require the capability to analyze large numbers of tumors for large numbers of potential mutations. Consider the p53 tumor suppressor gene, which is mutated in about half of all human tumors. The frequency of mutations varies for different tumors (See Fig. 2). More than 90 percent of mutations in the p53 gene are missense mutations that result in altered proteins and mutations have been detected in approximately 100 out of its 393 codons. Aside from the long and laborious process of direct sequencing, there are no easy methods for identifying the precise base change at a point mutation. Techniques for surveying genes for mutations have been developed in the last decade, including mutation specific oligonucleotide probing, chemical or enzymatic (RNase A) cleavage of base pair mismatches, allele specific PCR, and a mismatch amplification mutation assay [43-46]. However, each of these assays is limited because of the labor involved and number of gene sites that can be studied at one time. They also lack the sensitivity to identify mutations in a minority of cells in a sample. For these reasons researchers urgently need new, robust and sensitive methods of detecting cancer mutations.

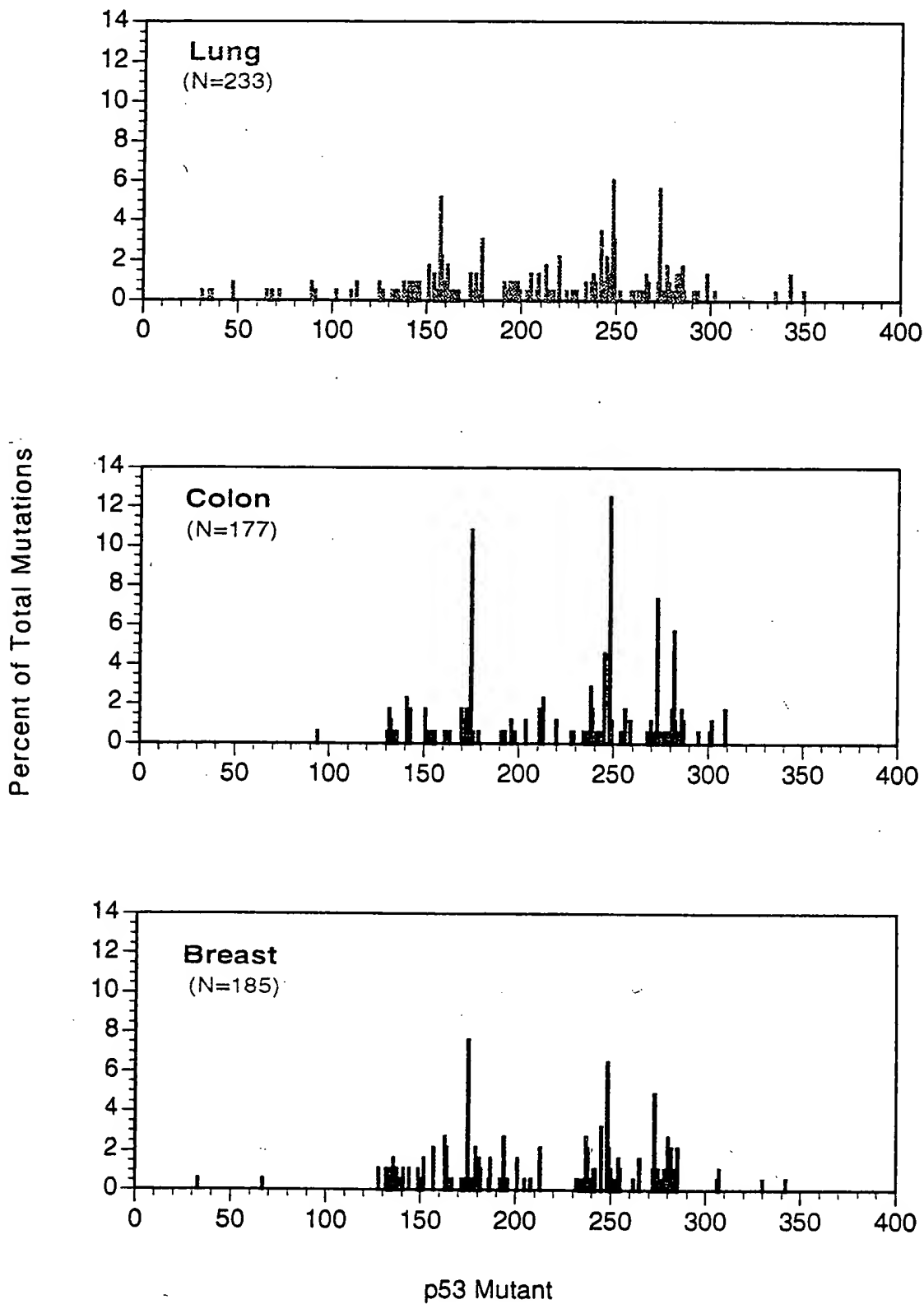


Fig. 2 Histograms showing frequencies of mutations at p53 gene codons. Adapted from [47, 48].

To meet this need for sensitive cancer detection methods, our long term objective is to develop these three technologies for characterizing genetic alterations:

- (i) **Polymerase chain reaction/ligase detection reaction (PCR/LDR).** This method amplifies defined DNA regions by PCR and detects the presence of mutations by LDR. PCR/LDR has the capacity to detect any of a large number of potential mutations simultaneously in a tumor sample with a sensitivity to identify one mutant DNA copy out of  $10^2$  to  $10^3$ . This method will eventually be used to detect dozens to hundreds of mutations in tumor tissue. It will also find use in detecting genetic and infectious disease.
- (ii) **Ligase detection reaction/polymerase chain reaction (LDR/PCR).** This method proportionally PCR amplifies ligase detection products. This technology will determine multiple gene deletions and amplifications in tumor specimens. It will also be used as a method for deletion mapping and detecting chromosomal abnormalities.
- (iii) **Polymerase chain reaction/restriction endonuclease/ligase detection reaction (PCR/RE/LDR).** This technique selectively amplifies mutant DNA sequence while removing wild type sequence. It has the sensitivity to detect one mutant cell in  $10^6$  to  $10^7$  normal cells. This method shows promise for finding micrometastases, when primary tumor mutations have been identified. It might also be of value for early disease detection by searching for cancer gene mutations in the blood, sputum, stool, and other specimens.

We wish to make it clear that the techniques we aim to develop is designed to survey tumors and tissues for mutations that have been discovered and described by other methods. We acknowledge the rapid advances being made in the detection of new oncogenic mutations [49]. An elegant method which amplifies and clones large and small differences between two genomes shows great promise for identifying genetic alterations associated with tumor development [50, 51]. We expect that mutations discovered by the other techniques would be added to panels of mutations that can be detected by our PCR/LCR and PCR/RE/LCR technologies.

When a new tumor mutation is discovered, one needs to determine: (i) whether the mutation is significant or simply a polymorphism; (ii) whether the gene plays a role in a specific cancer or cancers in general; and (iii) whether the mutation may be used diagnostically or prognostically. The methods we are developing will speed up the process of determining the clinical relevance of newly discovered mutations.

The process of evaluating mutations and developing our PCR/LDR and PCR/RE/LDR methods from basic research to clinical use is outlined in Fig. 3. These functions are served by several individual Projects and Cores in this program. For example, "Testing for detectable mutations" will be performed by Projects 1 and 2 in conjunction with Core B. (See Theme below.)

The low sensitivity mutation detection technique, PCR/LDR, will be used at different levels in the process of developing new methods of cancer detection. After it is technically feasible to test for a mutation, the clinical significance of that mutation can be determined for screening tests, diagnostic testing, or monitoring patients after treatment. For example, multiplex PCR/LDR could detect the presence of all high risk human papilloma virus strains, even in the presence of low risk strains, and thus potentially become a more sensitive and accurate method for predicting cervical cancer. All new DNA diagnostic methods must be appropriately evaluated and compared with currently accepted methods.

The high sensitivity mutation detection technique, PCR/RE/LDR, will be able to detect the presence of a few cancer cells in the presence of millions of normal cells. This may find immediate use in monitoring for recurrence of disease. The clinical significance of detecting mutations at this high sensitivity would have to be determined.

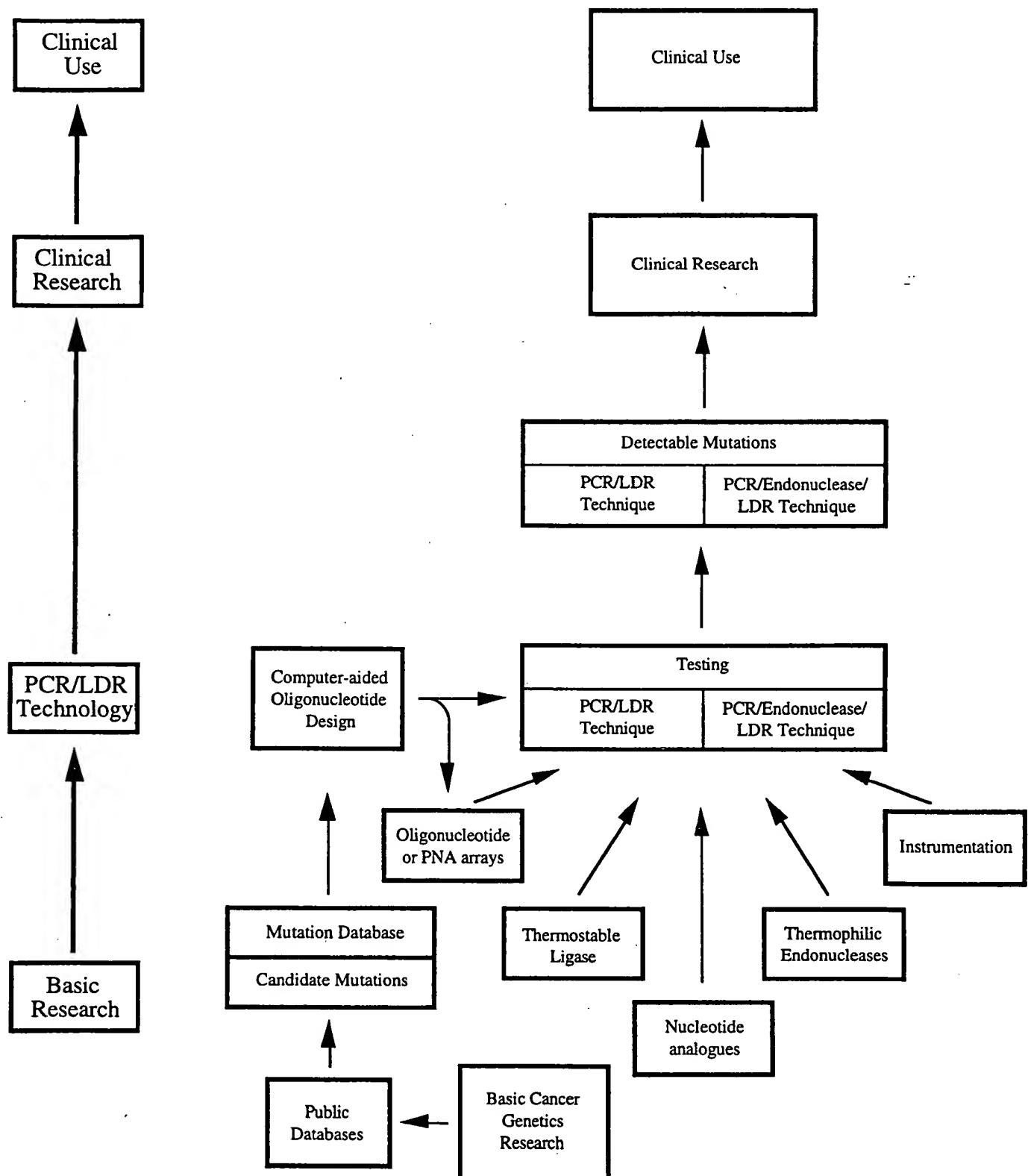


Fig. 3. Mutation evaluation and development in "New Methods for Cancer Detection"



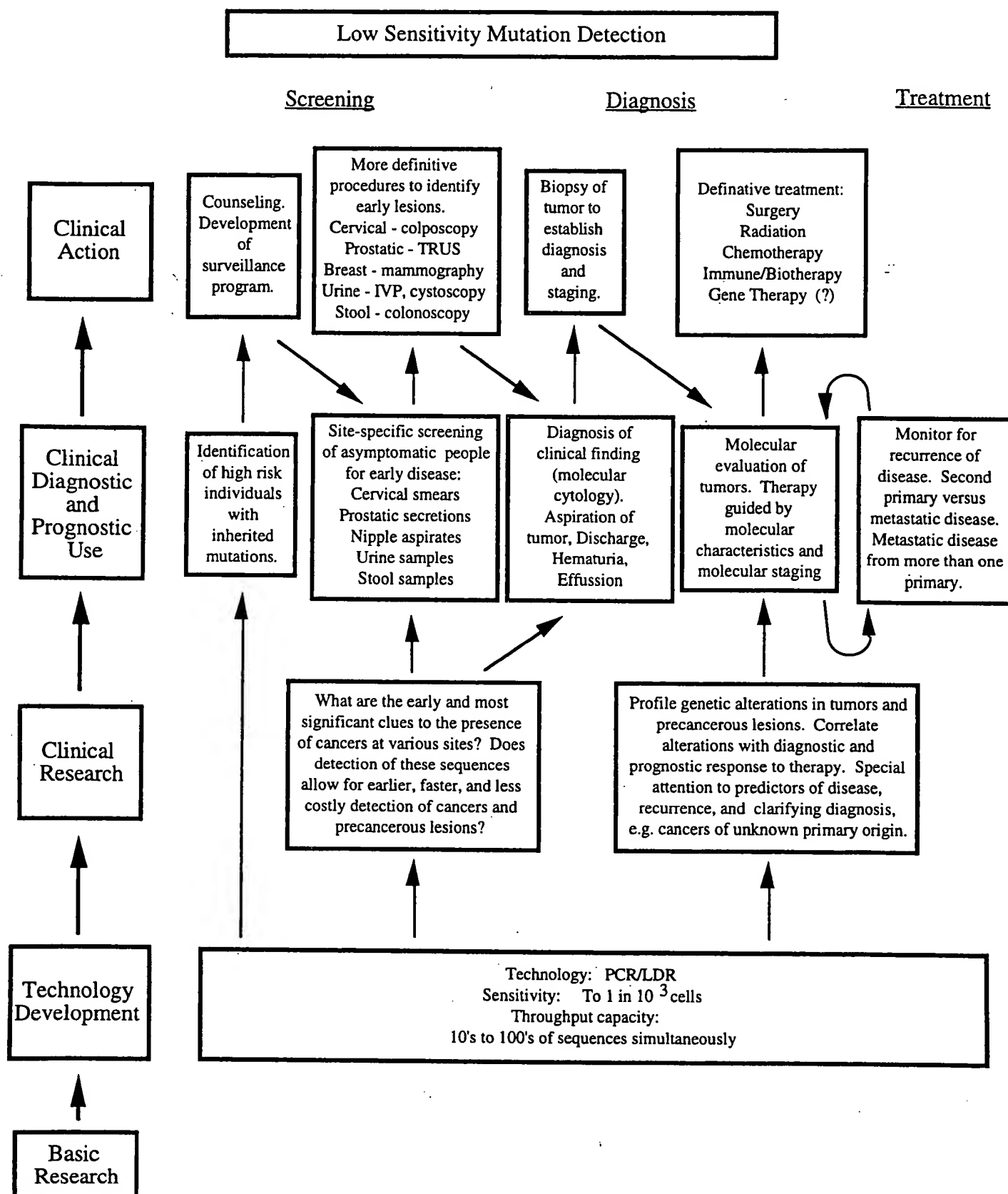


Fig. 4. Low sensitivity mutation detection. Flow chart showing stages of low sensitivity testing development.

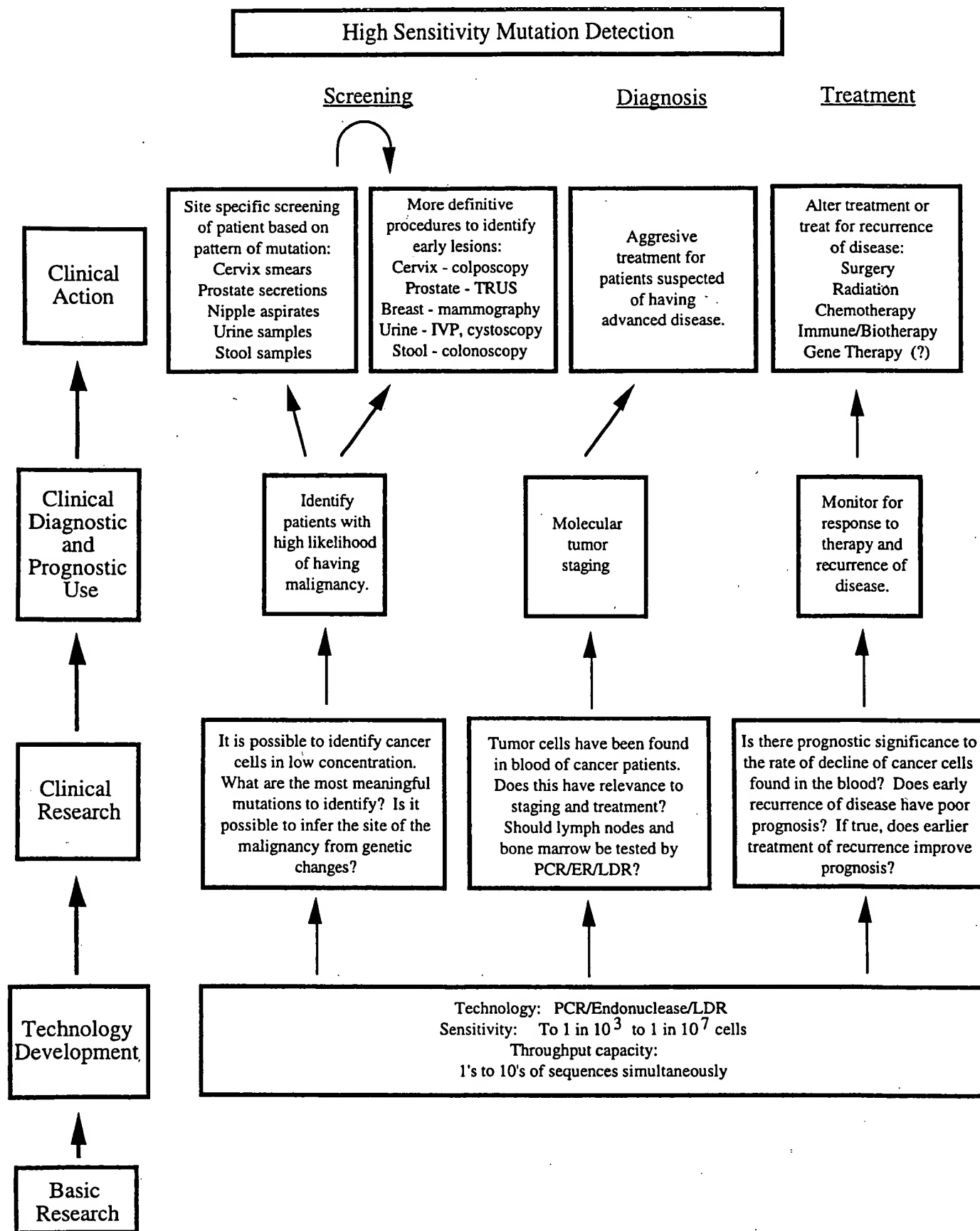


Fig. 5. High sensitivity mutation detection. Flow chart showing stages of high sensitivity testing development.

Table 1. Comparison of PCR/LDR to Current Methods of Disease Detection.

	<u>Issue</u>	<u>Disadvantage of current technology</u>	<u>Advantages of PCR/LDR technology</u>
<b><u>BACTERIA</u></b>			
<i>Mycobacterium tuberculosis</i>	Multiple drug resistance	Culture may require six months.	Can identify all allelic variants of rifampicin - and other drug - resistant genes. Allows treatment until organism clears from patient.
<i>K. pneumoniae</i> : β-lactamase resistant.	Resistance to broad spectrum antibiotics in some strains	Determining antibiotic resistance profile may take 48 hours or more.	Can identify penicillinase and pinpoint mutations causing resistance to most recent penicillins and cephalosporins. Change drug therapy.
<b><u>VIRUSES</u></b>			
Poliovirus	Revertant to wild-type virus during live vaccine production, can cause disease.	Difficulty in quantitation of precise amount of revertants.	Quantify exact portion of wild-type virus and correlate with virulence in monkeys. Will allow for improved control of vaccine production.
Human papillomavirus strains 16 and 18	Correlates with cervical cancers.	Difficulty in distinguishing between HPV16, 18 and non-carcinogenic strains.	Can accurately identify HPV16 and HPV18 in mixed infections. Allows for early diagnosis and preventive treatment.
<b><u>GENETIC DISEASES</u></b>			
21 Hydroxylase Deficiency	Presence of pseudogene causes high frequency of mutation.	Pseudogene mutations complicate analysis of genotype.	Accurate quantification of normal and pseudogene mutation will allow for rapid prenatal diagnosis and proper prenatal treatment.
Cystic fibrosis	Numerous mutations cause disease.	Current methods cannot easily screen hundreds of mutations.	PCR/LDR allows for simultaneous screening of many mutations. Will allow for accurate prenatal diagnosis.
<b><u>CANCER</u></b>			
Colon cancer	Early detection is costly (colonoscopy) and not well tolerated.	Molecular diagnostics of all <i>ras</i> mutations is not feasible on a large scale.	PCR/LDR will allow for detection of all <i>ras</i> mutations. Will identify patients for treatment by colonoscopy. Increased sensitivity and specificity at reduced cost.
Breast cancer	Screening is costly. Better prognostic indicators are needed.	Molecular methods for diagnosis and prognosis just now being explored.	PCR/LDR blood screening may be diagnostic. High throughput of PCR/LDR may accelerate correlation of genetic changes with clinical outcome.

We recognize that these technologies could help researchers, not only in the cancer field, but also in the areas of genetics and infectious diseases. Some of the potential benefits of PCR/LDR and PCR/RE/LDR are listed in Table 1. A list of collaborators who will explore the uses of this new technology is provided in Table 2, section G, and letters of collaboration follow.

## **B. Theme of the Program Project**

This program project application represents an integrated and focused multi-disciplinary approach to cancer, genetic and infectious disease detection. Our interactive team (Principal and co-Investigators, academic and industrial collaborators) spans the areas of molecular biology, clinical oncology, medical genetics, microbiology, biological and organic chemistry, protein engineering, structural biology, microfabrication engineering, and information science. We plan to take advantage of, and extend, recent advances in molecular biology and biotechnology which will allow the highly sensitive and specific identification of lesions in the DNA of cancer cells.

This program project has been divided into five projects and three cores which reflect the recommended format of a program project grant application. At the recommendation of the NCI program director, a Core was designated to include experiments which relate to projects 3 and 5. Due to the unusual nature of this proposal, it was suggested that we be comprehensive, and it was recognized that the individual Projects might go beyond the length of a standard RO1 application.

Projects 1 and 2 are parallel approaches to detecting cancer causing mutations, in lung and colon tumors (Project 1), or breast and cervix tumors (Project 2). These two projects will develop and evaluate the three technologies (PCR/LDR, LDR/PCR, and PCR/RE/LDR). Both teams will be evaluating tests for the most prevalent p53 tumor suppressor gene mutations in human cancer. Advances in one project will be immediately transferable to the other. These two projects will build on the accomplishments provided by the design and synthesis of nucleotide analogues (Project 3) which serve not only as "convertides", but also as universal base pairs. This will allow us to find mutations at any sequence using the PCR/RE/LDR high sensitivity technique. Basic research aimed at engineering an improved thermostable ligase (Project 4) may significantly increase the sensitivity of our PCR/LDR and other cancer detection methods. The ability to simultaneously detect hundreds of cancer mutations awaits the powerful new approaches to synthesizing addressable arrays (Project 5). Both the informatics support for cancer detection, and the instrumentation and mutation detection cores (Core A and Core B) play a critical role in achieving our goal of high sensitivity and specificity with multiplex cancer mutation detection.

One representation of the multiple interactions between the various projects and cores is shown in Fig. 6. These interactions are outlined below:

### **Project 1. Genetic Markers Of Lung And Colon Cancer.**

Project Leader:	Dr. V. Wilson
Collaborates with	Dr. F. Barany
Project 2	strong interactions, share ideas and procedures.
Project 3	provides primers containing internal or 3' nucleotide analogues for improved PCR/RE/LDR.
Project 4	provides improved thermostable ligase and ligation conditions for higher sensitivity.
Project 5	provides addressable arrays for higher throughput to Core B for use in Project 1.
Core A	provides informatic support, Project 1 provides results to Core A.
Core B	provides oligonucleotide synthesis and instrumentation support.

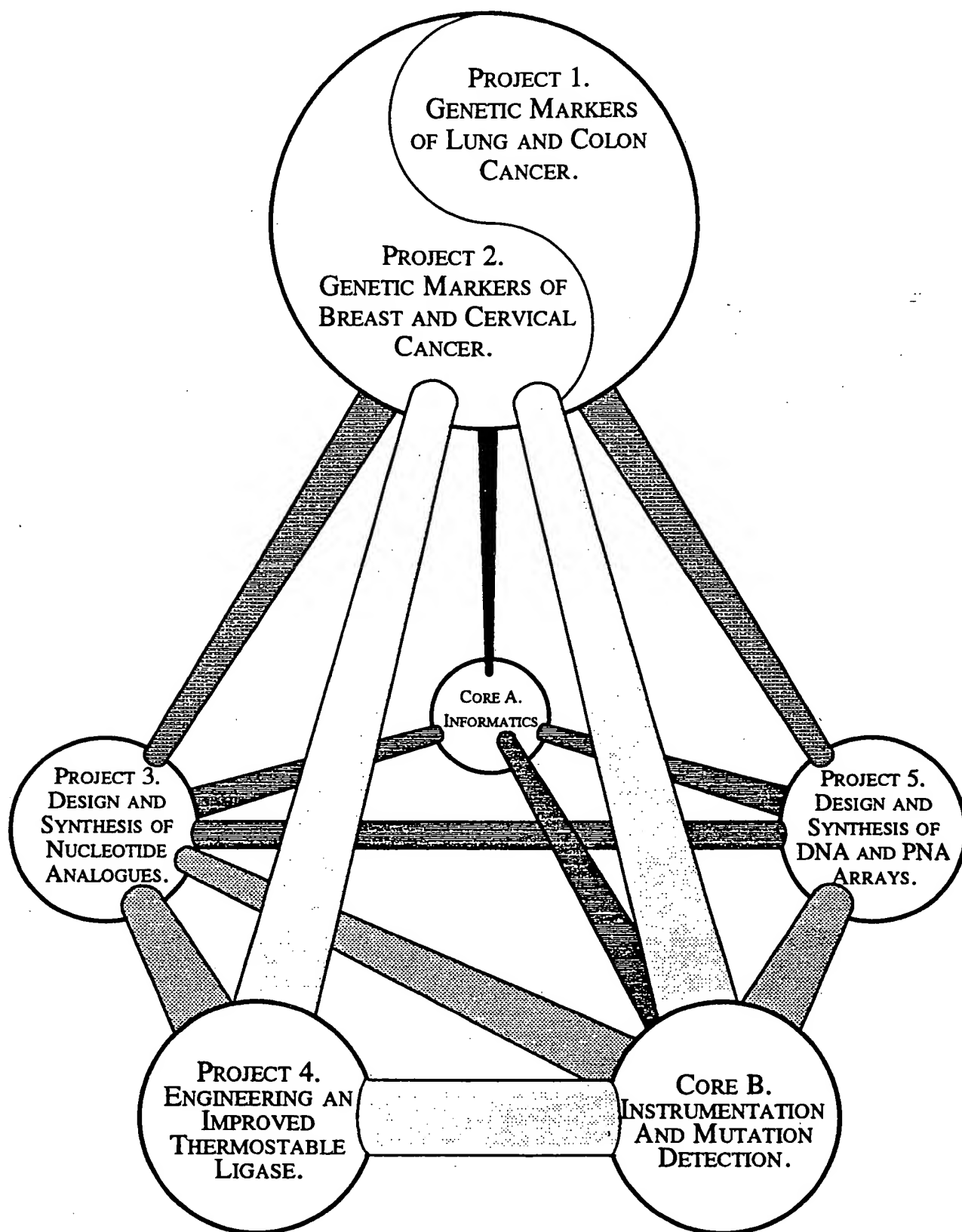


Fig. 6. Diagram of how the projects and cores interrelate.

**Project 2. Genetic Markers of Breast and Cervical Cancer.**

Project Leader: Dr. F. Barany  
Project Co-Leader: Dr. M. Lubin  
Project 1 strong interactions, share ideas and procedures.  
Project 3 provides primers containing internal or 3' nucleotide analogues for improved PCR/RE/LDR.  
Project 4 provides improved thermostable ligase and ligation conditions for higher sensitivity.  
Project 5 provides addressable arrays for higher throughput to Core B for use in Project 2.  
Core A provides informatic support, Project 2 provides results to Core A.  
Core B provides oligonucleotide synthesis and instrumentation support.

**Project 3. Design and Synthesis of Nucleotide Analogues.**

Project Leader: Dr. D. Bergstrom  
Project Co-Leader: Dr. R. Hammer  
Project 1 receives primers containing internal or 3' nucleotide analogues from Project 3.  
Project 2 receives primers containing internal or 3' nucleotide analogues from Project 3.  
Project 4 receives primers containing internal nucleotide analogues from Project 3.  
Project 5 receives nucleotide analogues for incorporation into DNA or PNA from Project 3.  
Project 5 strong interactions, share ideas  
Core A provides informatics support on primer design to Project 3.  
Core B provides results on efficiency and fidelity of polymerase extension off nucleotide analogue containing primers.

**Project 4. Engineering an Improved Thermostable Ligase.**

Project Leader: Dr. F. Barany  
Project Co-Leader: Dr. A. Aggarwal  
Project 1 receives improved thermostable ligase and ligation conditions from Project 4.  
Project 2 strong interactions, share ideas  
Project 2 receives improved thermostable ligase and ligation conditions from Project 4.  
Project 3 provides primers containing internal nucleotide analogues to Project 4.  
Core B provides oligonucleotide synthesis and instrumentation support.

**Project 5. Design and Synthesis of DNA and PNA Arrays.**

Project Leader: Dr. G. Barany  
Collaborates with Dr. R. Hammer  
Project 1 receives addressable arrays for higher throughput from Core B and Project 5.

Project 2 receives addressable arrays for higher throughput from Core B and Project 5.  
Project 3 provides nucleotide analogues for incorporation into DNA or PNA for Project 5.  
Project 3 strong interactions, share ideas  
Core A provides informatics support on DNA and PNA zip code design to Project 5.  
Core B tests addressable arrays synthesized in Project 5.

#### **Core A. Informatic Support for Cancer Detection Methods.**

Core Leader: Dr. N. Hackett  
Project 1 receives informatics support, and provides results to Core A.  
Project 1 receives informatics support, and provides results to Core A.  
Project 3 receives informatics support on primer design from Core A.  
Project 5 receives informatics support on DNA and PNA zip code design from Core A.  
Core B will be networked with Core A.

#### **Core B. Instrumentation and Mutation Detection.**

Core Leader: Dr. F. Barany  
Core Co-Leader: Dr. M. Lubin  
Project 1 receives oligonucleotide synthesis and instrumentation support from Core B.  
Project 1 receives oligonucleotide synthesis and instrumentation support from Core B.  
Project 3 receives results on efficiency and fidelity of polymerase extension off nucleotide analogue containing primers from Core B.  
Project 4 receives oligonucleotide synthesis and instrumentation support from Core B.  
Project 5 prepares addressable arrays for further testing by Core B, and use in Projects 1 and 2  
Core A will be networked with Core B.

#### **Core C. Administrative Core.**

Core Leader: Dr. F. Barany  
Core Co-Leader: Dr. M. Bunk  
Core C provides administrative support to all Projects and Cores.

### **C. RESEARCH PLAN**

The long range objective of our research is to develop sensitive and specific approaches to the detection and simultaneous identification of cancer-related, genetic alterations. Mutations and genetic aberrations have been implicated, at various steps, in the etiology and biology of tumors. Somatic mutations in tumor suppressor genes, oncogene amplification and viral DNA sequences have been found in cancers. However, the clinical use of these discoveries and research into their clinical significance has been slowed by the laborious processes by which they are detected. To apply these discoveries and explore the interactions of multiple genetic alterations, we urgently need new technologies, which are capable of being automated and have the power to detect any of a vast number of mutations. We have assembled a team of investigators whose expertise will be directed toward innovative solutions to this problem.

Our strategic approach to this problem will be to:

- (i) Develop a multiplex polymerase chain reaction/ligase detection reaction (PCR/LDR) system for the detection of somatic mutations in tumors.
- (ii) Develop a ligase detection reaction/ polymerase chain reaction (LDR/PCR) system for the detection of gene amplifications and deletions in tumors.
- (iii) Develop the capability to detect and identify mutations in rare cancer cells at a sensitivity of 1 in  $10^6$  or 1 in  $10^7$  with a PCR/restriction enzyme/LDR (PCR/RE/LDR) system.
- (iv) Design and synthesize nucleotide analogues for converting specific DNA sequences into restriction endonuclease recognition sites.
- (v) Engineer a thermostable ligase with greater specificity.
- (vi) Develop methods for the simultaneous detection of multiplex LDR products using oligonucleotide or peptide nucleotide arrays.
- (vii) Explore the ability of these technologies to further our understanding and clinical management of lung, colon, breast and cervical cancers.

**Project 1** will develop PCR/LDR, and explore a variation on this, PCR/LCR for detection of *K-ras* and *p53* mutations in colon and lung tumors. In addition, PCR/RE/LCR will be used to explore the background level of somatic mutations at certain loci. The same technique will be used to identify micrometastases in blood and other clinical specimens.

**Project 2** will develop PCR/LDR for detection of *p53* mutations in breast tumors and human papilloma virus sequences in cervical cancers. Using DNA from trisomic individuals, LDR/PCR will be developed and then applied to breast tumor specimens to investigate *Her-2/neu* gene amplification. In addition, PCR/RE/LCR will be used to explore bone marrow and lymph nodes for micrometastases.

**Project 3** will design and synthesize nucleotide analogues which facilitate sequence conversion for use with PCR/RE/LDR. "Convertides" are nucleoside analogues which pair to one or more of the natural bases in an initial primer hybridization. More importantly, convertides also function as a degenerate template allowing for insertion of a different base during subsequent rounds of polymerase amplification.

**Project 4** will develop assays for increasing the specificity of *Tth* ligase. Site-specific mutants of this enzyme will be constructed and tested for increased fidelity. Determining the three-dimensional structure of *Tth* ligase should help us understand the mechanism of enzyme action, as well as design additional mutants.

**Project 5** will develop solid-phase approaches for the simultaneous detection of LDR products. Products will be captured on a spatially addressable array, so that the position of a signal identifies a mutation. Each LDR product will have a "zip code" tail, which will be captured selectively by a "complementary zip code" on the solid support. Multiple reuse of a universal "complementary zip code" array is envisaged to allow detection of a wide range of cancers and genetic diseases.

*Sequence of events leading to the collaboration and current group effort.*

The collaborations described in this program project application evolved as follows: Shortly after publication of the paper in which Dr. F. Barany described the ligase chain reaction (Proc. Nat. Acad. Sci. 88:189-193), he received a phone call from Dr. V. Wilson who pointed out that the method could be used for cancer detection. An extensive discussion ensued concerning the biochemistry and specificities of restriction enzymes, ligases, and polymerases, and a strategy for looking at mutations in the *H-ras* gene was mapped out. The new strategy was similar to restriction selection approaches reported by others, but with the key difference that final product detection would be by LCR. Neither of us imagined that the added LCR step would push the detection sensitivity to one mutant allele in  $10^8$  wild-type alleles [52-54]. This was about 1,000 fold more sensitive than the standard selection procedures, and immediately assured us that *Taq* polymerase fidelity



would not interfere with exquisitely sensitive detection of cancer mutations. (For other approaches, see [55-64]). As experimental results from this collaboration were being generated in Dr. Wilson's laboratory throughout 1992, Dr. F. Barany started to consider ways to generalize the method. A breakthrough occurred in January 1993, with an idea for using the restriction enzyme *TaqI* to detect all CG dinucleotides, which account for 40% of the mutations reported in the p53 tumor suppressor gene [65]. Over the next few months, a further generalization was outlined for detecting hundreds of mutations at a low sensitivity level of one in  $10^2$  to  $10^3$  (PCR/LDR), and a few mutations at high sensitivity level of one in  $10^6$  to  $10^7$  (PCR/RE/LDR). In March 1993, Dr. F. Barany visited Dr. James Jacobson of the National Cancer Institute to present the plan and spell out the various components that would be required to bring it to fruition. Dr. Jacobson encouraged Dr. Barany to carry out further preliminary experiments, and at the same time to consider organizing a program project grant application. From these discussions, it became clear that it would be necessary to assemble a team of collaborating scientists with expertise in oncology, nucleic acid chemistry, X-ray crystallography, polymer-supported chemistry, and information processing.

To identify co-investigators for a program project application, Dr. F. Barany consulted with numerous scientific colleagues in academia and industry. One approach was to solidify ongoing collaborations and scientific relationships for the requirements of cancer detection; in this way Dr. V. Wilson, Dr. M. Lubin, Dr. A. Aggarwal, and Dr. N. Hackett were asked to participate. The various chemistry challenges were discussed extensively with Dr. G. Barany, who recommended that Dr. R. Hammer and Dr. D. Bergstrom be invited to join the program project. The synergy of this nucleus of co-investigators led to substantial new insights on how to implement the original vision.

A follow-up meeting with Dr. Jacobson occurred in August 1993, by which time Dr. F. Barany had shown successfully that the generalized high sensitivity cancer detection scheme worked. At the same time Dr. F. Barany met with Dr. R. Strausberg and other program officers at the National Center for Human Genome Research. From these meetings it became clear that the main goal of the research would be to develop new DNA detection tools which could ultimately improve cancer care. Both Dr. Jacobson and Dr. Strausberg foresaw the potential that this approach would also have on detecting genetic and infectious diseases. During these meetings and in subsequent correspondence it was decided that a program project grant would be submitted for primary review by the National Cancer Institute, but the grant should also include a list of collaborators in related fields who would benefit from this work. This explains in part the over 30 academic, government, and industrial collaborators who have joined in our effort. (Please see letters of collaboration, and Table 2, section G, summarizing their research interests and the projects with which they interact).

In several cases, there have been prior collaborative relationships among investigators on the program project team. Most relevant to the theme of the program project is the three-year collaboration between Dr. F. Barany of Cornell University Medical College and Dr. V. Wilson of The University of Colorado School of Medicine, as already discussed. Furthermore, Dr. F. Barany and Dr. A. Aggarwal of Columbia University have been collaborating over the past four years on the crystallization and structure determination of the *TaqI* restriction enzyme, hence providing a firm basis for the proposed studies on the *Tth* ligase. Dr. F. Barany helped to recruit Dr. N. Hackett. Over the past three years they have frequently discussed the role of informatics in molecular biology. Dr. M. Lubin works across the street at the Strang Cancer Prevention Center, and over the past year, has helped Dr. F. Barany learn about the cancer field in general. Dr. Lubin specifically formulated the LDR/PCR idea for quantifying chromosomal gene copies. On the chemistry team, Dr. D. Bergstrom and Dr. G. Barany have known each other for some twenty years, dating back to a mentor-student relationship at The Rockefeller University at New York. More recently, Dr. R. Hammer received his doctoral training with Dr. G. Barany at the University of Minnesota. By virtue of his postdoctoral work at the ETH in Zurich, Dr. R. Hammer is uniquely positioned to integrate the diverse chemistry aspects (peptides, nucleic acids, solid-phase synthesis) that must be brought together for success in the program project. Finally, Dr. F. Barany and Dr. G. Barany, having attended the same elementary school, Junior High School, High School, and Graduate School (G. Barany skipped college), and having shared scientific ideas during their entire professional careers are as close as ... brothers.

**D. PRELIMINARY STUDIES**

In 1991, Dr. F. Barany developed the ligase chain reaction (LCR), a powerful method for detecting single base mutations. In this assay *Tth* ligase discriminates between normal and mutant DNA and amplifies the sequence [66, 67]. This enzyme links two oligonucleotides when they anneal adjacently on a complementary target sequence at 65°C. A mismatch at the junction of the two oligonucleotides prohibits ligation thus distinguishing between two DNA sequences. The ligated product can serve as a template for another set of oligonucleotides, complementary to the first pair. If these anneal adjacent to each other on the product of the first ligation, they too will be ligated and a ligase chain reaction will proceed exponentially with thermal cycling. (See Fig. 7) Using only one pair of adjacent primers gives a linear amplification called ligase detection reaction (LDR). LCR and LDR are compatible with PCR and allow the multiplex detection of single base mutations without primer interference.

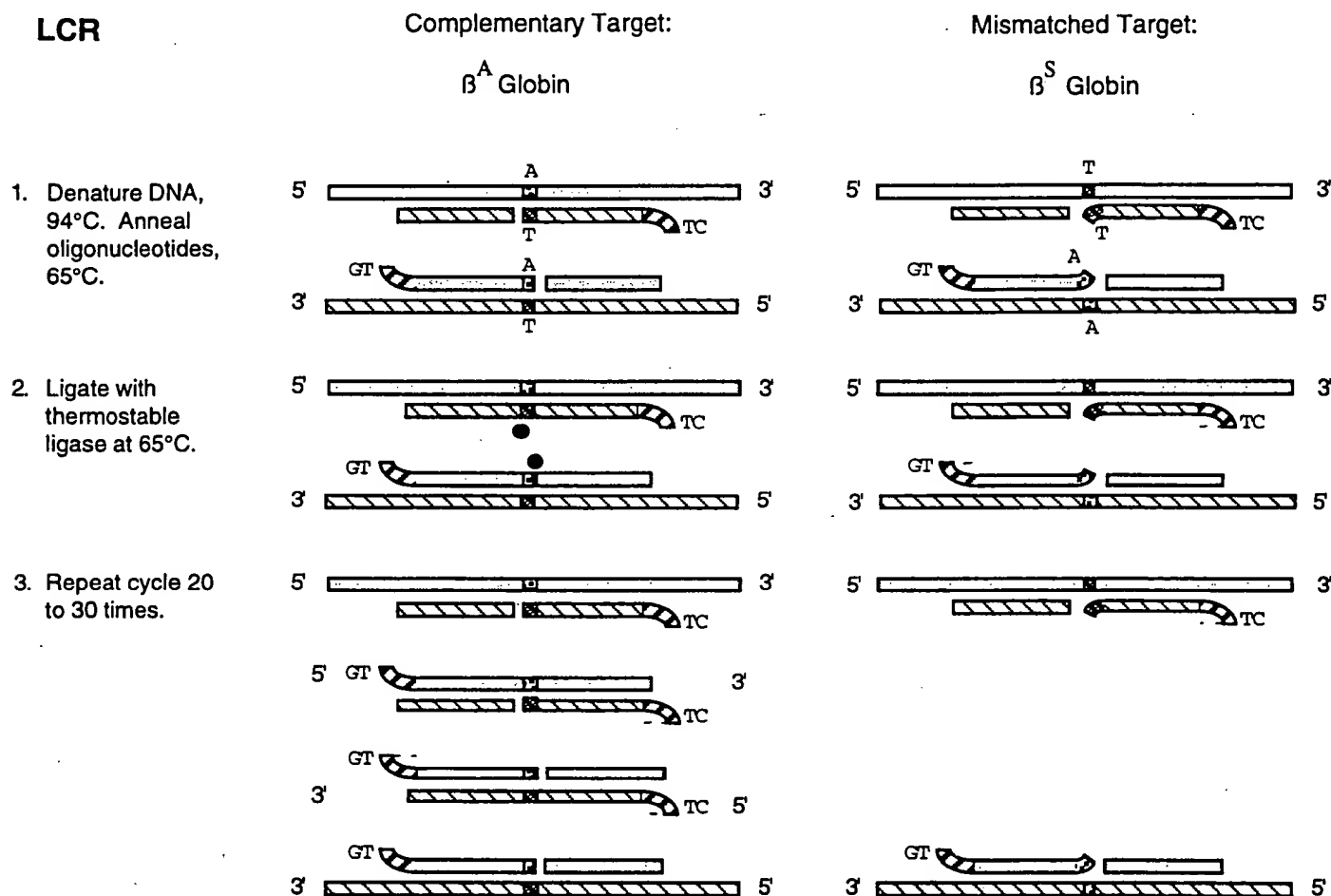


Fig. 7. Allele-specific DNA amplification and detection using the ligase chain reaction (LCR). DNA is denatured at 94°C and the four LCR primers anneal to their complementary strands at 65°C, near their melting temperatures ( $T_m$ ). Thermostable ligase (depicted as a black circle) will only ligate adjacent primers that are perfectly complementary to their target sequences. On the left a complementary target ( $\beta^A$ ) allows ligation. A single base-pair mismatch at the internal 3' end of the adjacent primers prevents ligation (as shown in the case of  $\beta^S$  on the right). A pair of primers complementary to  $\beta^S$  could be used to detect this mutation. The discriminating primers have non-complementary GT or CT tails at their 5' ends to avoid ligation in the wrong orientation.

The ligase chain reaction is ideal for multiplexing. Since there is no polymerization step, several primer sets can ligate along a gene without interference. The optimal multiplex detection scheme involves a primary PCR amplification, followed by either LCR (using four primers for exponential amplification) or LDR (using two primers for linear amplification). This PCR/LDR approach has been successfully applied to multiplex

detection of 30 different cystic fibrosis mutations [68-70], hyperkalemic periodic paralysis [71], and 21 hydroxylase deficiency (D. Day, P. White, and F. Barany, unpublished). In the 21 hydroxylase study, individuals are determined to be heterozygous or homozygous for any of the ten common gene conversions that cause that disease (see Fig. 8). Dr. V. Wilson has used PCR/LCR to directly detect a *K-ras* mutation in a colonic polyp.

## PCR/ LDR

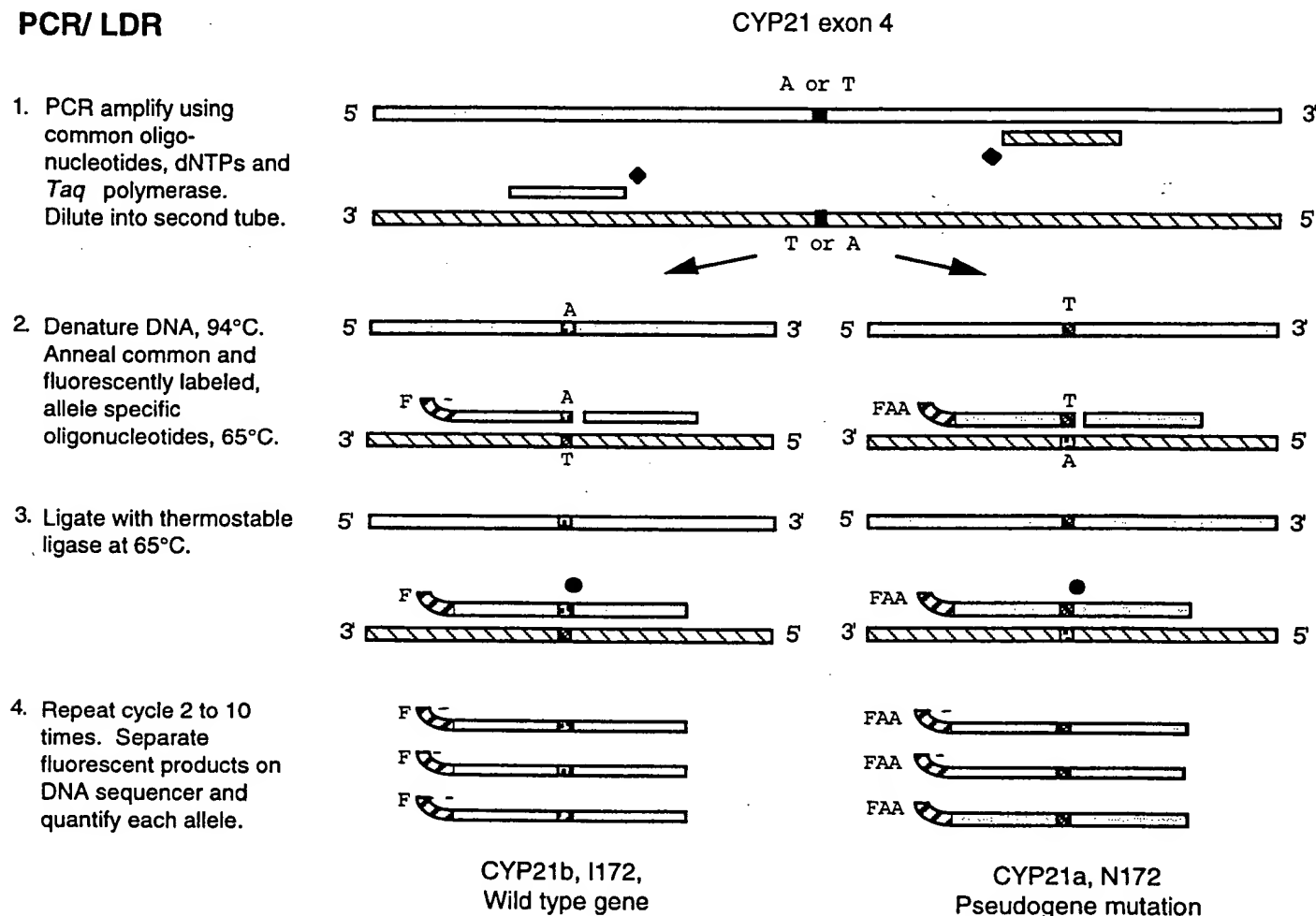
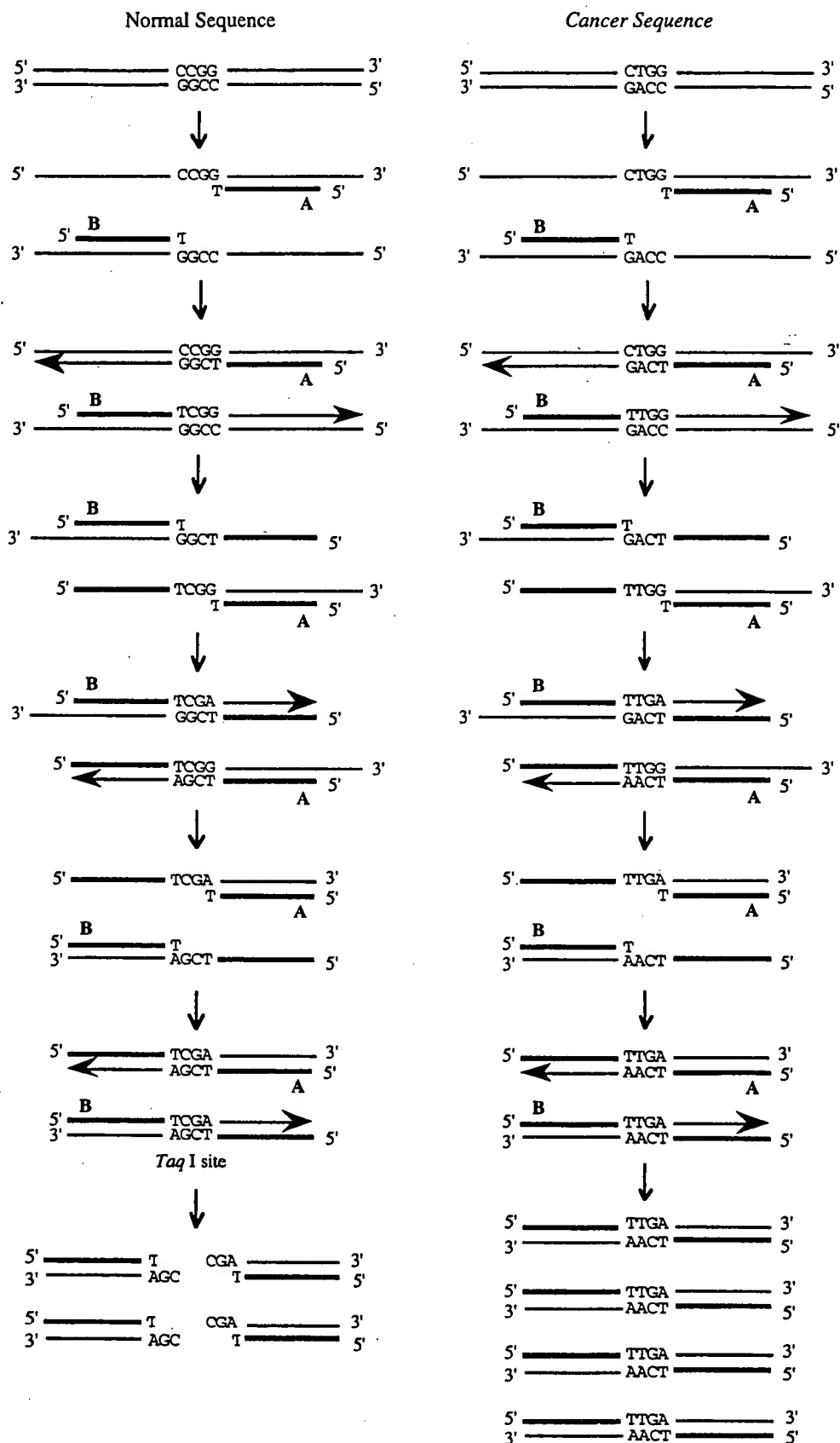


Fig. 8. Allele-specific detection and quantification using the polymerase chain reaction with the ligase detection reaction (PCR/LDR). The fragment of interest is amplified by a primary PCR reaction using *Taq* polymerase (depicted as a black diamond). DNA is denatured and two LDR primers anneal to their complementary strands. The allele specific LDR primers have discriminating bases on their 3' ends and different length "tails" at their 5' ends. The allele-specific LDR primers will only ligate to the adjacent common primer when there is no mismatch at the junction. If the common primer is limiting, the allele specific products generated are proportional to the alleles present in the starting target DNA. In this example PCR is used to amplify exon 4 in the CYP21 (steroid 21 hydroxylase) gene. Allele-specific primers contain fluorescent groups at their 5' ends on different length ploy-A tails. The discriminating bases are at their 3' ends. Equimolar ligation of both allele specific primers indicates the individual is heterozygous. PCR/LDR may be used in a multiplex format, in which 30 primers can distinguish 20 individual alleles in the same reaction.

In a collaboration between Dr. V. Wilson and Dr. F. Barany a simple PCR amplification /endonuclease selection /LCR detection method (PCR/RE/LCR) has been developed to achieve even high sensitivity of mutation detection. Preliminary studies in the *Ha-ras* gene have demonstrated detection of 10 mutated copies in  $10^9$  wild-type sequences [52-54]. (Similar approaches of PCR/endonuclease detection of *Ha-ras* gene mutations give less sensitivity since they lack the LCR detection step [58-64]). Dr. V. Wilson has now extended this high sensitivity for detecting mutations in the p53 tumor suppressor gene at codon 248.

**PCR/ RE/ LDR**

1. Denature DNA at 94°C.
2. Anneal primers A and B.  
Both primers contain a T at their 3' end.
3. Extend with *Taq* Polymerase at a dNTP's concentration of 800 uM.
4. Denature DNA at 94°C.  
(Only newly synthesized DNA shown - for clarity.)
5. Anneal primers A and B.
6. Extend with *Taq* Polymerase.
7. Denature DNA at 94°C.  
(Only newly synthesized DNA shown - for clarity.)
8. Anneal primers A and B.
9. Extend with *Taq* Polymerase.
10. Normal DNA is cut by *Taq* I endonuclease. Cancer DNA is not cut, and continues to amplify.



**Fig. 7. A generalized method to detect and identify mutations at a sensitivity of 1 in  $10^6$  or  $10^7$  cells. See text on next page.**

We now have developed a generalized method for converting *every* pre-existing CG dinucleotide sequences in a cancer-causing gene into a PCR product containing a *TaqI* restriction endonuclease site (T↓CGA) at that position (See Fig. 7). PCR is used to amplify a two base region in both the majority normal and minority cancer DNA. By judicious design of the primers, a new restriction site is created within the normal--but not the mutated DNA. The two PCR primers are perfectly complementary to the DNA flanking the CG dinucleotide, *except* for a 3' T nucleotide, independent of whether it matches the actual nucleotide flanking the CG dinucleotide (See Fig 7, step 2). By using very rapid PCR amplifications at high dNTP concentrations, this mismatched T is extended [59, 72]. After the first round of amplification each primer generates a sequence of the form TCGN (Step 3). Since the second round of amplification uses the complementary primer, this sequence is converted into TCGA, the *TaqI* site that was sought (Step 5). A cancer mutation (TG) would be converted to TTGA sequence, which is refractory to *TaqI* cleavage. During a second set of PCR amplifications, normal DNA is cleaved by three reiterative additions of a thermophilic restriction endonuclease, in this example *TaqI* (Step 10). A final LDR step detects the actual cancer causing mutation [66]. The ligase detection step allows for accurate quantification of the amount of original cancer mutation, as well as avoiding false positive signals from primer dimers. This concept has been generalized to include 9 restriction endonucleases, which can detect all 16 possible dinucleotide pairs, and hence any cancer mutation at 1 in 10<sup>6</sup> sensitivity.

The completely generalized method of cancer detection requires converting any dinucleotide sequence into a restriction site. The efficiency of this reaction will be vastly improved by using nucleotide analogues which exhibit relaxed base-pairing. Dr. D. Bergstrom has designed and synthesized such a "convertide" which we have designated **Q2** (3-nitropyrrole 2'-deoxyribonucleoside). Primers containing this convertide were able to act as both specific sequencing primers, as well as specific PCR primers.

The ability to simultaneously detect hundreds of cancer mutations awaits the powerful new approach of the addressable array. Dr. G. Barany has pioneered the development of numerous protecting groups and resins required in solid-phase peptide synthesis. One of his recent projects involves preparing "shaved" beads, in which synthesis of the desired peptide is directed only to the surface of the bead. This will be valuable for one of our approaches to synthesizing addressable arrays.

*The following is a synopsis of the qualifications of all of the Co-Investigators in the program.*

Dr. Francis Barany received his Ph.D. in Microbiology in 1981 at The Rockefeller University with Dr. Alexander Tomasz. He was a Helen Hay Whitney postdoctoral fellow with Dr. Hamilton O. Smith at the Johns Hopkins University School of Medicine from 1982-1985. Upon appointment as an Assistant Professor in Microbiology at Cornell University Medical College in 1985, he was named a Cornell Scholar in Biomedical Sciences. He currently holds the rank of Associate professor and was awarded a five year Hirschl/Monique Weill-Caulier Career Scientist Award in 1992. He is best known for cloning thermostable ligase and developing the ligase chain reaction (LCR) for the detection of genetic diseases.

Dr. Anel K. Aggarwal received his Ph.D. in Biophysics from Kings College, University of London. He conducted his postdoctoral studies in the laboratory of Dr. Stephen C. Harrison at Harvard, where he determined the high resolution structure of phage 434 repressor-DNA complex by X-ray crystallography. Dr. Aggarwal is currently Assistant Professor of Biochemistry and Molecular Biophysics at College of Physicians and Surgeons of Columbia University. His laboratory continues to study the three dimensional structures of DNA binding proteins by X-ray crystallography. He is regarded as one of the foremost experts on the crystallization of DNA binding proteins with oligodeoxynucleotides. His laboratory has recently determined the high resolution structures of restriction endonuclease *BamHI*, with and without DNA.

Dr. George Barany received his Ph.D. in Chemistry in 1977, and was a postdoctoral fellow at The Rockefeller University with Dr. Bruce Merrifield. Dr. Barany is currently Professor of Chemistry at the University of Minnesota. Dr. Barany's research career has focused on the development of new methods for solid-phase peptide synthesis, including orthogonal protection strategies, handles (linkers) to supports, and polymeric supports. His pioneering contributions have been widely recognized, most recently when he received the prestigious Vincent du Vigneaud Award. Several reagents, derivatives, and supports introduced

through Dr. Barany's research efforts have become commercially available from Millipore Corporation and other companies, and are used in laboratories throughout the world.

Dr. Donald E. Bergstrom received his Ph.D. in Chemistry in 1970 from the University of California Berkeley with Professor Henry Rapoport. Following postdoctoral research with Dr. Nelson Leonard at the University of Illinois in 1970-71, he spent two years in the laboratory of William Agosta at the Rockefeller University. He is currently Walther Professor of Medicinal Chemistry at Purdue University. Dr. Bergstrom directs a research group that includes three postdoctoral fellows and seven graduate students in the Department of Medicinal Chemistry and Pharmacognosy at Purdue. Dr. Bergstrom is best known for his research in the area of modified nucleoside synthesis. He was a pioneer in the synthesis of C-5 modified pyrimidine nucleosides, which have been important tools for molecular biology research.

Dr. Neil Hackett obtained his Ph.D. in biochemistry in 1982 from the University of British Columbia, and was a postdoctoral fellow with Gobind Khorana at the Massachusetts Institute of Technology. He was Assistant Professor of Molecular Biology at Vanderbilt University from 1986, until coming to Cornell Medical College in 1989 as an Assistant Professor of Microbiology. His research interests have focused on molecular biology of bacteria including *E. coli*, *Halobacteria* and recently *Mycobacterium tuberculosis*. He has recently completed physical mapping of the *Halobacterium* genome which involved computer programming for image analysis and data manipulation. He has also managed and administered the DNA sequence analysis computing facility since 1990, providing the service for the whole campus.

Dr. Robert P. Hammer received his Ph.D. in chemistry in 1990 from the University of Minnesota with Professor George Barany. He spent two years at the Swiss Institute of Technology (ETH) as a postdoctoral fellow with Professor Albert Eschenmoser. Dr. Hammer is currently an Assistant Professor of Chemistry in the Department of Chemistry at Louisiana State University. He has a broad knowledge of organic and biological chemistry. His specific expertise in nucleotide and peptide chemistry will be valuable assets as the co-leader of Project 3 (Nucleotide Analogues) and as a collaborator on Project 5 (Addressable Arrays). His most notable contributions, include development of orthogonal protection schemes for solid-phase peptide synthesis and *de novo* synthesis of hexose-based nucleosides and their oligomers (as yet unpublished).

Dr. Matthew Lubin received his M.D. in 1984 and completed his residency in internal medicine in 1987 at the University of Medicine and Dentistry of New Jersey-New Jersey Medical School, Newark. He completed a postdoctoral fellowship in the Division of Medical Genetics at UCLA School of Medicine with Drs. David Rimoim and Elizabeth Neufeld in 1990, where his studies focused on genetics of common adult disorders. He is currently the Director of Medical Genetics at the Strang Cancer Prevention Center, where he oversees several clinical and research programs in cancer genetics. He holds the rank of Assistant Professor in the Department of Medicine at The New York Hospital-Cornell University Medical Center.

Dr. Vincent Wilson received his Ph.D. in pharmacology in 1980 at Oregon State University, and subsequently trained with Dr. Curtis Harris at the National Cancer Institute. He is currently the Director of the Molecular Genetics/Oncology Laboratory in the Department of Pathology at The Children's Hospital in Denver, Colorado, and Associate Professor of Pathology at the University of Colorado School of Medicine, University of Colorado Health Sciences Center, Denver. Dr. Wilson's research work in the mechanisms of carcinogenesis, DNA damage, and DNA 5-methyl-deoxycytidine patterns is well recognized. Over the last five years, Dr. Wilson has built and established a certified clinical genetic testing service (DNA diagnostics) laboratory that focuses on oncological diseases. He is presently involved in laboratory studies of cancer families as a member of the team that established an Hereditary Cancer Clinic in Denver. His research studies in mechanisms of carcinogenesis and in the early detection of cancer has lead to a coupling of efforts with Dr. F. Barany for the development and advancement of the extremely sensitive mutation detection methods that are the foundation of this proposal.

*List of publications and manuscripts accepted, which have been produced by collaborators.*

Wei, Q., Barany, F., & Wilson, V.L.: Sensitive detection of point mutations by combined PCR and LCR Techniques. *Proceedings of the International Conference on Molecular Biology of Genetic Diseases*, Shanghai, P.R. China, pp. 40, (1992).

- Wei, Q., Barany, F., & Wilson, V.L.: Oncogenic point mutations detected by combined PCR and LCR Techniques. *Mol. Biol. Cell* 3 (supplement): 22a (1992).
- Wilson, V.L., Wei, Q., Parker, N., Manchester, D.K., & Barany, F.: Frequency of oncogenic mutations in human tissues determined by combined PCR and LCR techniques. *Proc. Am. Assoc. Cancer Res.* 34: 262 (1993).
- Fernando Albericio, Nancy Kneib-Cordonier, Lajos Gera, Robert P. Hammer, Derek Hudson and George Barany, "Solid-Phase Synthesis of Peptide Amides Under Mild Conditions," In *Peptides - Chemistry and Biology: Proceedings of the Tenth American Peptide Symposium* (G.R. Marshall, ed.), Escom Science Publishers, Leiden, The Netherlands, 1988, pp. 159-161.
- C. García-Echeverría, R.P. Hammer, M.A. Molins, F. Albericio, M. Pons, G. Barany and E. Giralt, "Cyclization of Disulfide-Containing Peptides in Solid-Phase Synthesis," In *Peptides - Chemistry and Biology: Proceedings of the Eleventh American Peptide Symposium* (J. Rivier & G.R. Marshall, eds.), Escom Science Publishers, Leiden, The Netherlands, 1990, pp. 996-998.
- R.P. Hammer, F. Albericio, L. Gera and G. Barany, "Practical Approach to Solid-Phase Synthesis of C-Terminal Peptide Amides Under Mild Conditions Based on a Photolysable Anchoring Linkage," *Int. J. Peptide Protein Res.* 36, 31-45 (1990).
- F. Albericio, R.P. Hammer, C. García-Echeverría, M.A. Molins, J.L. Chang, M.C. Munson, M. Pons, E. Giralt and G. Barany, "Cyclization of Disulfide-Containing Peptides in Solid-Phase Synthesis," *Int. J. Peptide Protein Res.* 37, 402-413 (1991). C.R. Johnson, S. Biancalana, R.P. Hammer, P.B. Wright, and D. Hudson, "New Active Esters and Coupling Reagents Based on Pyrazolinones," In *Peptides - Chemistry and Biology: Proceedings of the Twelfth American Peptide Symposium* (J. Smith & J. Rivier, eds.), Escom Science Publishers, Leiden, The Netherlands, 1991, pp. 585-586.
- D. Hudson, C.R. Johnson, G. Barany, S. Biancalana, B.J. Calnan, A.D. Frankel, W.B. Cohn, T. Hayes, C. Dahl, M.A. Markus, M.A. Weiss, R.P. Hammer, H.-t. Hsu, R. Jordan, K.K. Kamo, M.H. Lyttle, L. Toll, D.S. Tsou, and P.B. Wright, "Tactics and Strategies in Solid-Phase Peptide Synthesis: New Directions, Methods and Applications," In *Innovations and Perspectives in Solid Phase Synthesis and Related Technologies: Peptides, Polypeptides, and Oligonucleotides 1992* (R. Epton, ed.), Intercept, Andover, England, 1992, pp. 135-152.

## E. INSTITUTIONAL ENVIRONMENT AND RESOURCES

The research for this program project grant comprises five projects and three cores, and takes place at seven sites. Three of these sites are in close physical proximity; Cornell University Medical College and the Strang Cancer Prevention Center are across the street from one another on the East side of Manhattan, while the College of Physicians & Surgeons of Columbia University is in uptown Manhattan. During the past few years, cross country and international scientific collaborations have become commonplace, largely due to the widespread use of Fax machines and Internet. We have a very effective communication network among all of the co-investigators who now routinely communicate through, phone, Fax, E.mail, and computer disks via Express mail.

Cornell University Medical College, New York. Dr. Francis Barany's laboratory consists of 670 sq. ft., which is part of 3,500 sq. ft. of research space he shares with Dr. William Holloman in the Department of Microbiology. Dr. Barany has an ABI 394 DNA Synthesizer which will be used for oligonucleotide synthesis and an ABI 373 automated fluorescent DNA sequencer with Genescan 672 software for DNA analysis and quantification. This equipment will serve as the initial equipment for Core B, the instrumentation core of this program. The laboratory is well equipped for the molecular biological engineering of improved thermostable ligase (Project 4). Addition resource assets include extensive computerization, access to facilities at



Rockefeller University and Memorial Sloan-Kettering Cancer Center and proximity to the other collaborators at Cornell, Strang and Columbia.

The DNA sequence analysis computing facility is managed by Dr. Neil Hackett and consists of three pieces of equipment. A Phosphorimager (Molecular Dynamics) provides quantitative autoradiography for routine molecular biology experiments. A Lynx5000 workstation (Applied Imaging) provides quantitative analysis of ethidium bromide stained gels. And a SPARCstation 2 has the Genetics Computer Group package of programs for retrieval and analysis of protein and DNA sequences. The system is networked and has 50 users on the CUMC campus.

College of Physicians & Surgeons of Columbia University, New York. The laboratory of Dr. Aggarwal is located in the Department of Biochemistry and Molecular Biophysics, at the Health Sciences Campus of Columbia University. His laboratory shares the Columbia X-ray Diffraction Facility with one other crystallographer. The Diffraction facility contains 2 Rigaku X-ray generators; 2 cameras, and a Xuong-Hamlin area detector. The laboratory is also well equipped for biochemical work, and has access to state of the art computational facilities.

The Children's Hospital and University of Colorado Health Sciences Center, Denver. Dr. Wilson's laboratory is located in the Department of Pathology and the Research Center of The Children's Hospital, where he is the director of a clinical oncology genetic testing service. For the last several years he has conducted research which is directly related to this proposal. The Denver academic environment is well suited for the clinical research to be conducted in Project 1. An experienced pathologist, Dr. Miller is the Director of the University of Colorado Cancer Center Tissue Core Laboratory. Dr. Franklin is an experienced pathologist and the Director of the University of Colorado SPORE Lung Cancer Tissue Bank Core I Laboratory. Dr. Kennedy is the Medical Director of the Lung Cancer Institute of Colorado, is an experienced bronchoscopist, and the Co-Director, with Ms. Proudfoot, of the University of Colorado SPORE Lung Cancer Tissue Procurement and Sputa Bank Core II Laboratory. These facilities, located at the University of Colorado School of Medicine, collect specimens from the entire Mountain States Region.

Louisiana State University, Baton Rouge. Dr. Hammer's laboratories are located in the Department of Chemistry on the main campus of Louisiana State University in Baton Rouge. All equipment for performing the organic synthesis of nucleoside analogs (Project 3) as well as the DNA and PNA propynyl monomers (Project 5) are already present in these laboratories. Oligonucleotides will be assembled on the Pharmacia Synthesizer in Dr. Hammer's laboratories. Essential support facilities such as NMR and mass spectrometry are housed in the same building and are readily accessible to researchers on this project.

Purdue University, West Lafayette, IN. Dr. Bergstrom's research group occupies a recently constructed laboratory (completed May 1993) in the Hansen Life Sciences Research Building at Purdue University. Ten researchers occupy a laboratory of 2500 sq. ft., complete with a central instrumentation room and ten hoods for synthetic chemistry operations. One section of this laboratory consisting of 24 linear feet of bench space, three hoods and three desks, will be dedicated to the current project. Dr. Bergstrom's office (150 sq. ft.) contains a conference table is located in the center of the laboratory. Instrumentation available to Dr. Bergstrom's group is listed in the facilities section of the Project 3 proposal.

Strang Cancer Prevention Center, New York. More than 5,000 patients a year visit the Strang Cancer Prevention Center, located near the New York Hospital-Cornell Medical Center. Breast cancer-related visits for screening, pre- and post-surgical evaluations, and chemotherapy total 4,000 a year. The Strang-Cornell Laboratories with 4,500 sq. ft. of space are well equipped and located near by. Approximately 320 sq. ft. of laboratory space is dedicated to Dr. Lubin's research. Strang's Director, Dr. Michael Osborne, is Chief of the Breast Surgery Service at the New York Hospital-Cornell Medical Center. For this project he has graciously offered us access to his tissue bank consisting of fresh frozen tumor specimens on over 600 breast cancer cases, with accompanying blood and bone marrow samples on more than 200.

University of Minnesota, Minneapolis. Dr. G. Barany's laboratories are located in the Department of Chemistry on the Minneapolis campus. These are well equipped to carry out the proposed work. Supporting resources in the Department and elsewhere on campus are an additional strength. Synthesis and



characterization of oligonucleotide and PNA monomers and oligomers, as well as of a wide variety of solid supports needed for synthesis and hybridizations, will be done by Dr. Barany's team at the University of Minnesota. Additional contributions will be made by industrial collaborators.

## F. ORGANIZATION AND ADMINISTRATIVE STRUCTURE

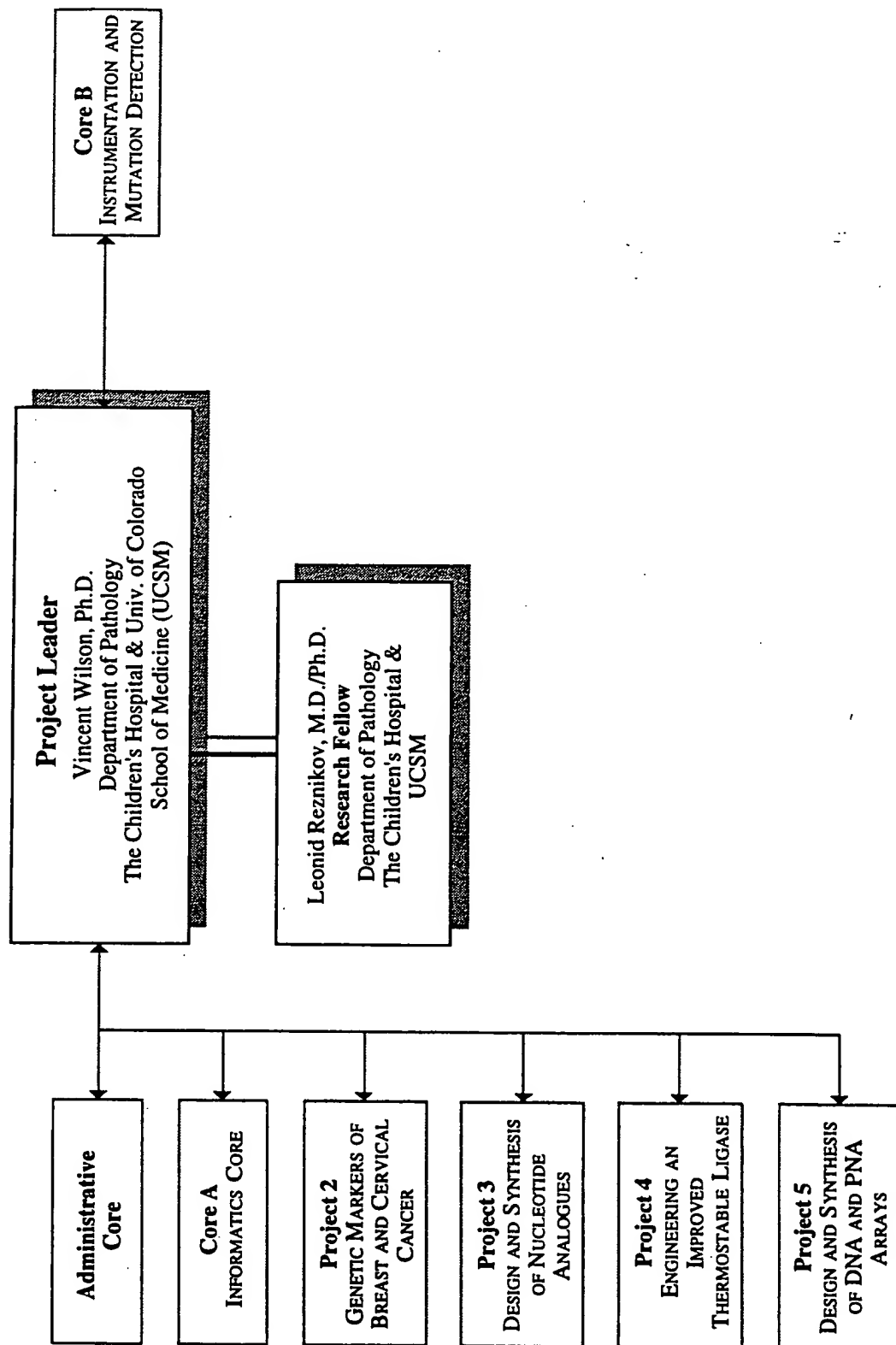
Dr. Francis Barany, as Principal Investigator will be responsible for all research activities, for encouraging scientific interchange between team members, and for coordinating activities with the NCI scientific staff. Dr. F. Barany will be assisted by Dr. Michael Bunk, who will serve as core director of the Administrative Core.

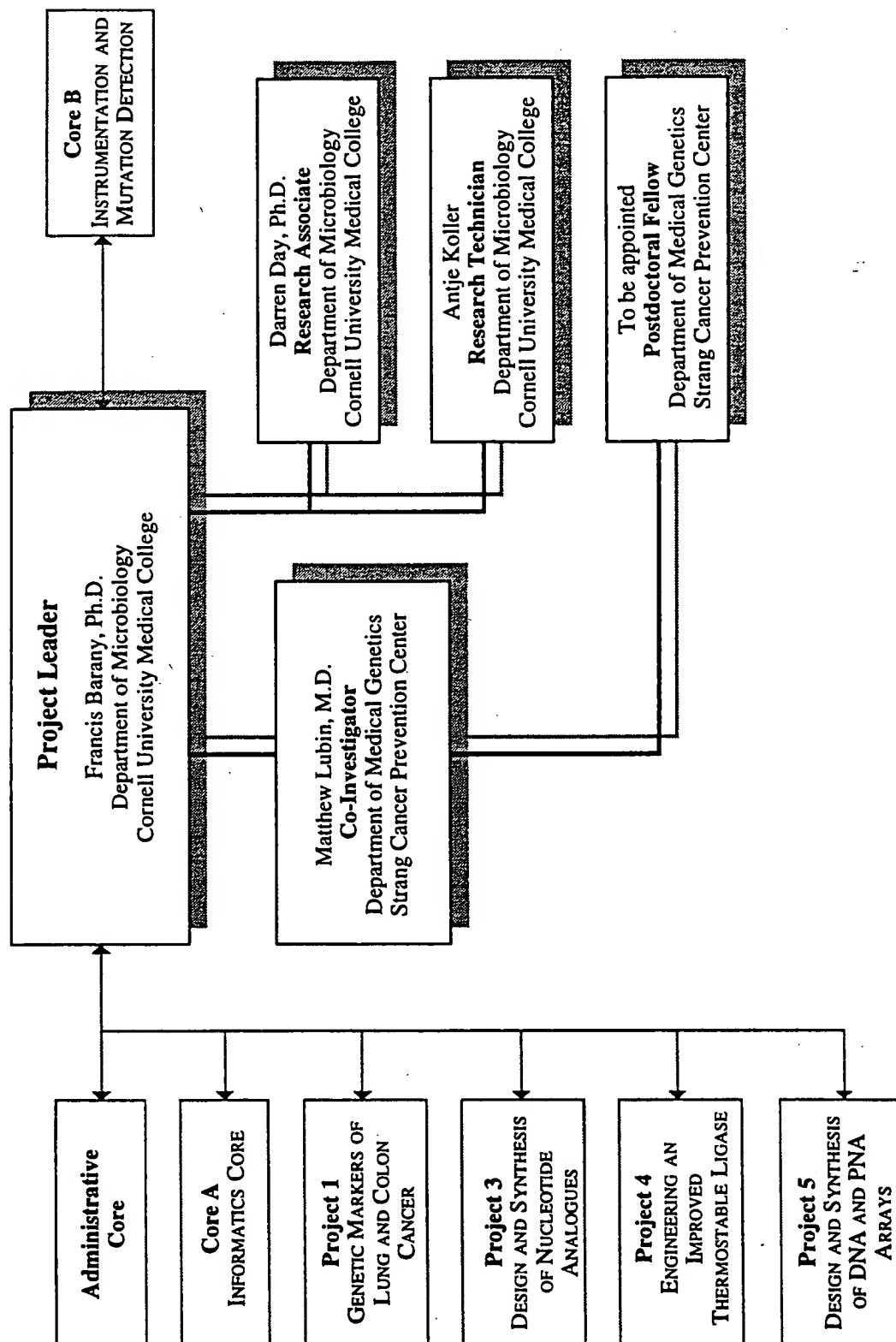
The unique feature of this program project is the synergistic interaction between all the projects and team members (see Fig. 4). Advances in one project will have an immediately beneficial effect on many of the other projects. While a part of the whole, each project can proceed as an independent unit, and does not *require* a particular enzyme or reagent from another project in order to proceed. This organizational structure can be likened to parallel processing, which has considerable advantages over the more conventional linear chains of command and communication.

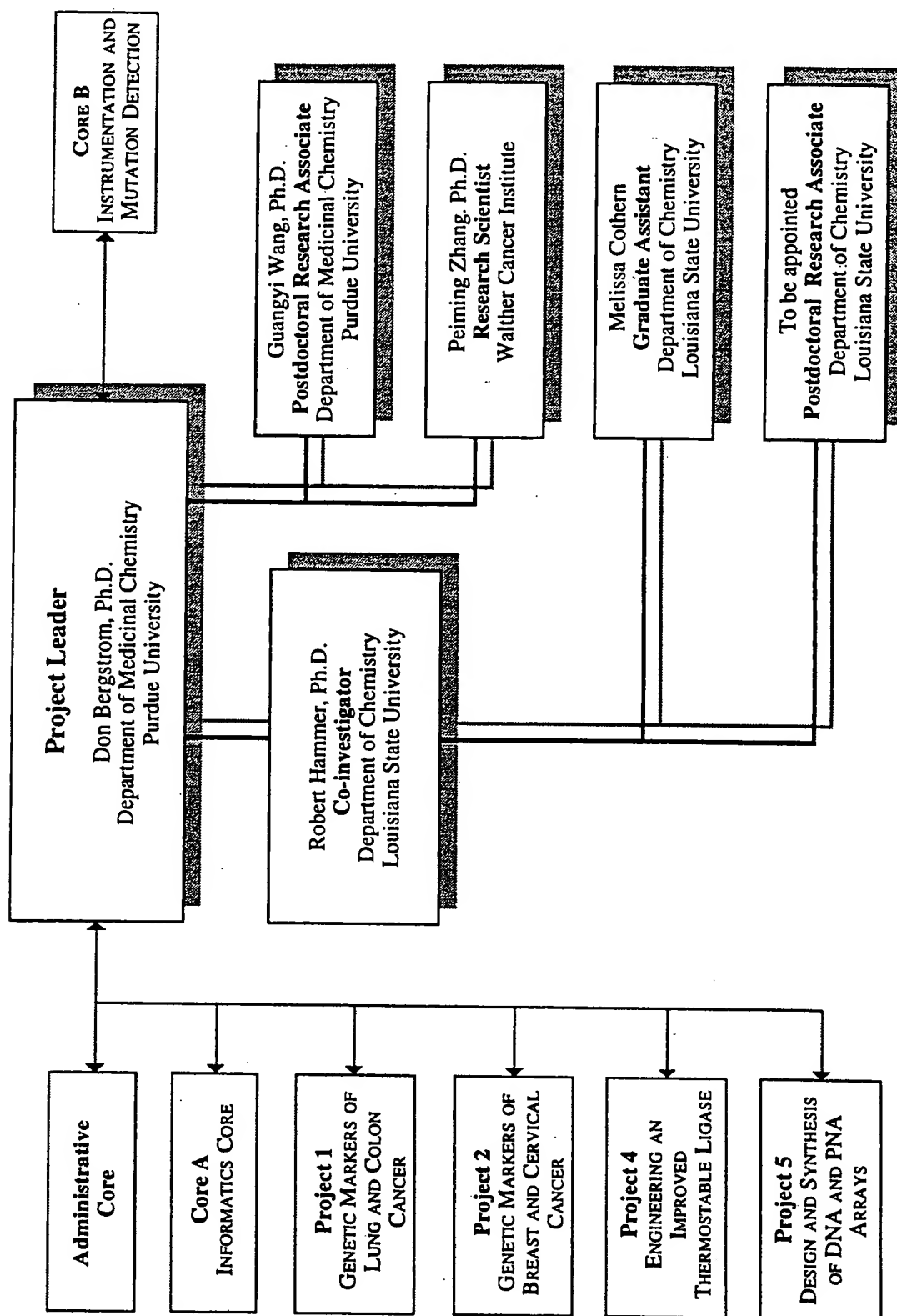
The accompanying diagrams show the decision making processes in each of the Projects and Cores. Each Project leader will be responsible for co-ordinating his co-workers on the projects. The diagrams do not adequately picture the interactive collaborations already ongoing between the projects and co-investigators. All co-investigators share the common goal of developing new methods for cancer and disease detection and could be considered collaborators on each other's projects. The diverse background and fields of expertise among the co-investigators provides a unique opportunity for cross-fertilization of ideas. For example the concept of addressable arrays composed of PNA oligomers assembled from tetramer building blocks arose from late night discussions between Dr. F. Barany, Dr. G. Barany, and Dr. R. Hammer. These discussions started as an exploration into use of DNA polymers composed of dinucleotide repeat sequences as a way of targeting a bifunctional zipcode to its correct address. The concept of quantifying gene duplications and deletions originated from discussions on amplifying very weak LDR product signals between Dr. Mathew Lubin and Dr. F. Barany. Finding the optimal DNA sequences to implement this concept required Dr. Neil Hackett's computer expertise.

All Co-investigators agreed to have annual program meetings alternating between New York City, and Bethesda. Since both collaborators and co-investigators come from diverse backgrounds, it is important that there are annual meetings. These meetings will serve not only as a means of presenting progress among the individual labs, but also as a means of furthering our understanding of the molecular mechanics of cancers. With this in mind, we plan to invite two internationally renowned experts in cancer or genetic diseases to each meeting. These experts may be from our current collaborators (see section G), or outside. For the first year, we are considering inviting Dr. John Kovach from the Mayo Clinic, Dr. Steven Friend from Harvard Medical School, or Dr. Mark Sobel from the National Cancer Institute. We would also like to invite local experts, for example Dr. Larry Norton and Dr. Jerard Hurwitz from Memorial Sloan Kettering Cancer Institute for meetings in New York City, or Dr. Curtis Harris of the National Cancer Institute for meetings in Bethesda. Invited experts will evaluate our work and make critical suggestions for improving our research. In addition, these experts would be invited to bring us up to date on their work. These experts could subsequently become members of an internal advisory group. We recognize that our current approach is narrowly focused on evaluating mutations in the p53 tumor suppressor gene and *ras* oncogene. As the technology improves, internal advisors will guide our approaches with regard to clinical relevance. Organization of the annual program meeting will be handled by the administrative core program coordinator.

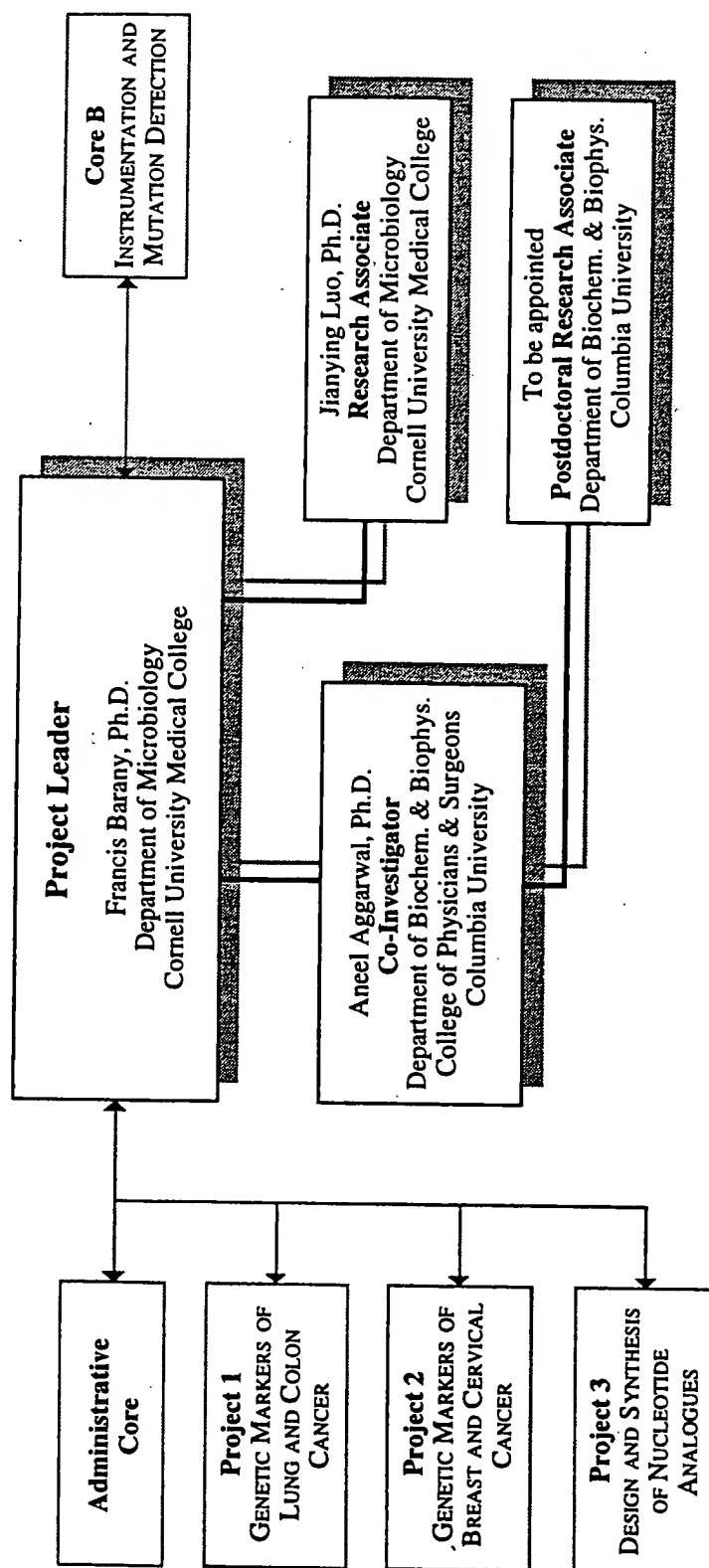
We would also favor forming an external advisory group which could advise us at other times. Program members would submit annual progress reports to such an advisory group, for confidential evaluation and suggestions. External advisory group members would be invited to the annual meeting. We will seek the advice of NCI staff scientists in forming such a group.

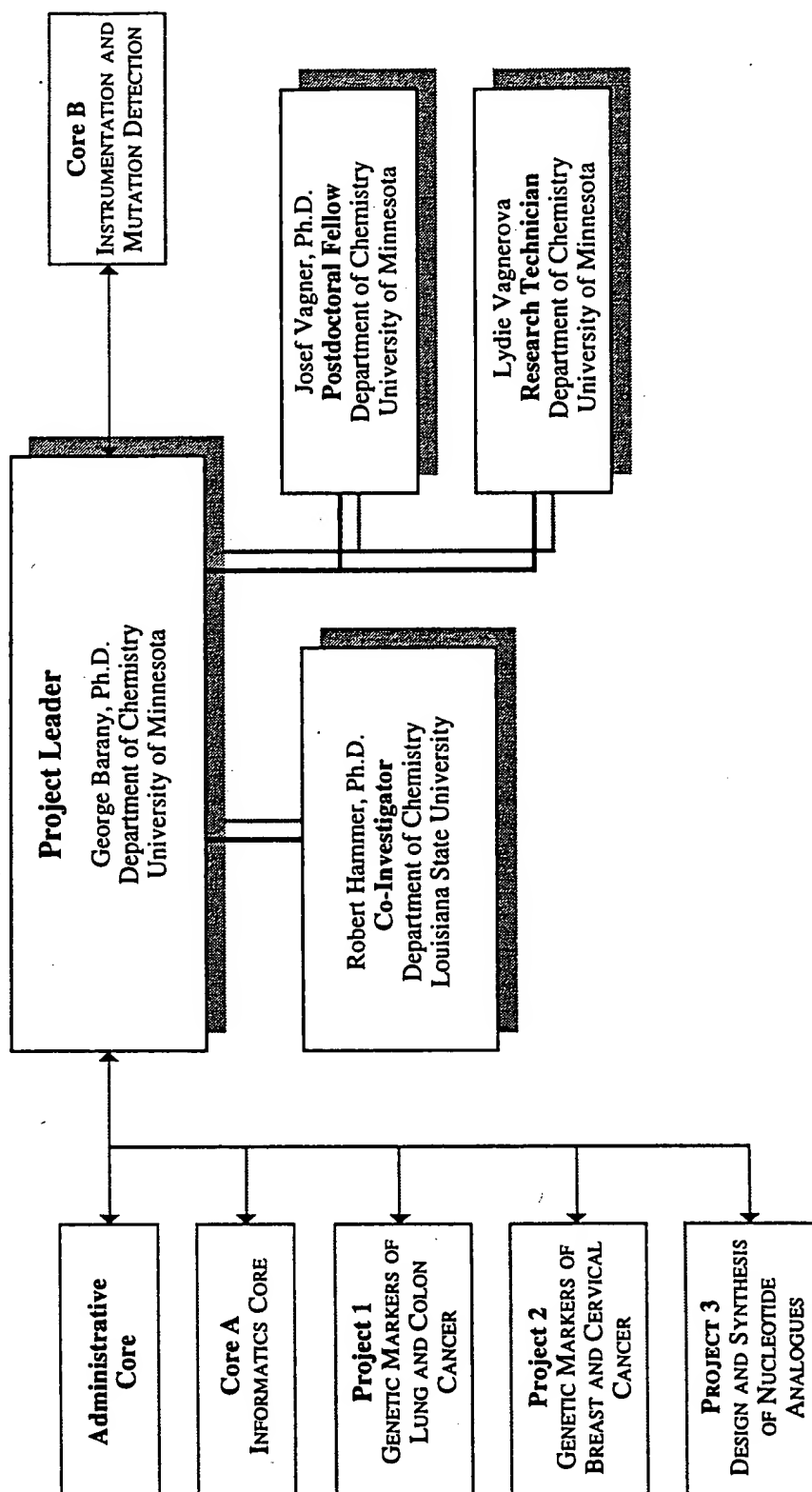
**Project 1 Genetic Markers of Lung and Colon Cancer**

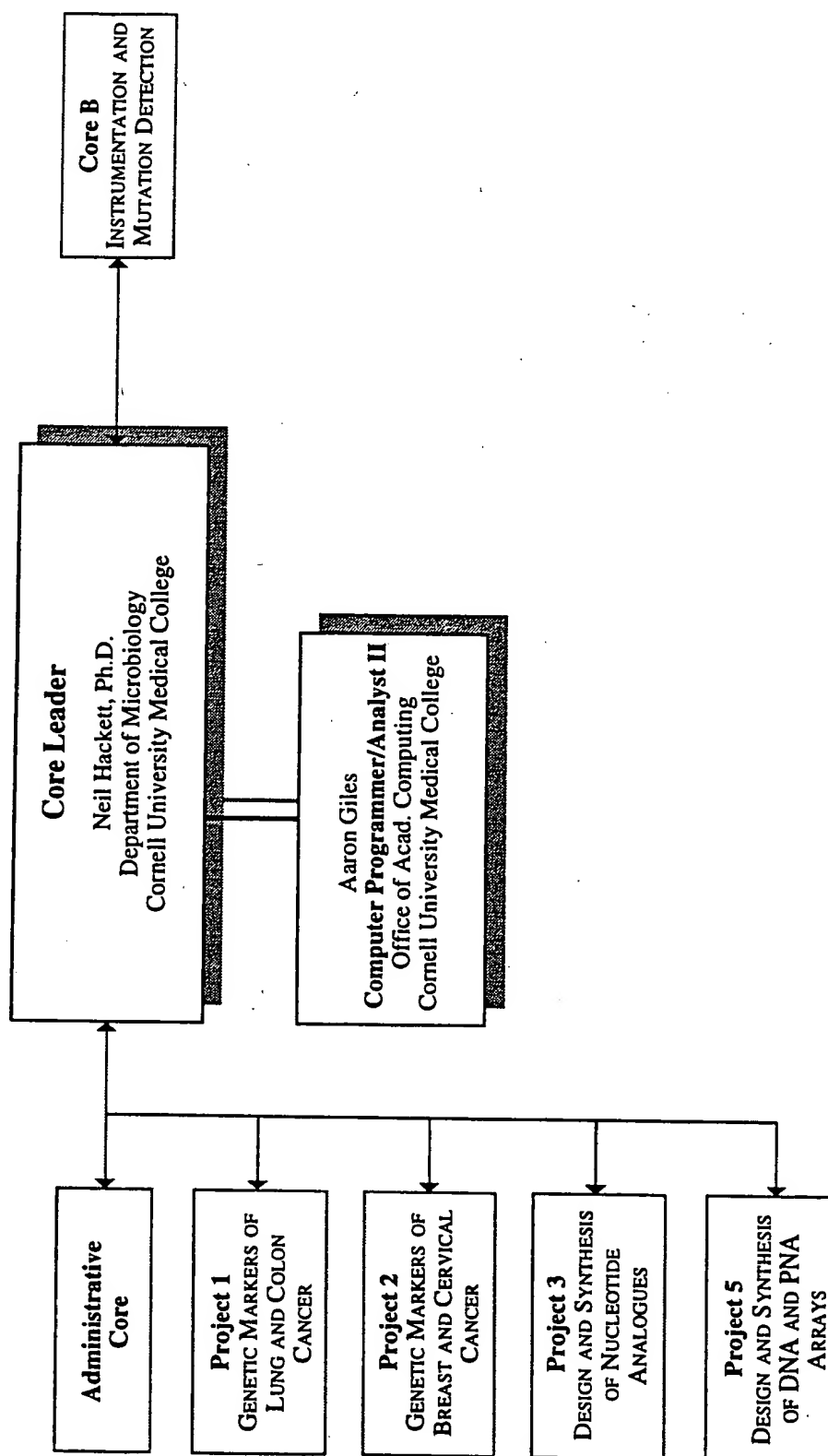
**Project 2 Genetic Markers of Breast and Cervical Cancer**

**Project 3 Design and Synthesis of Nucleotide Analogues**

## Project 4 Engineering an Improved Thermostable Ligase

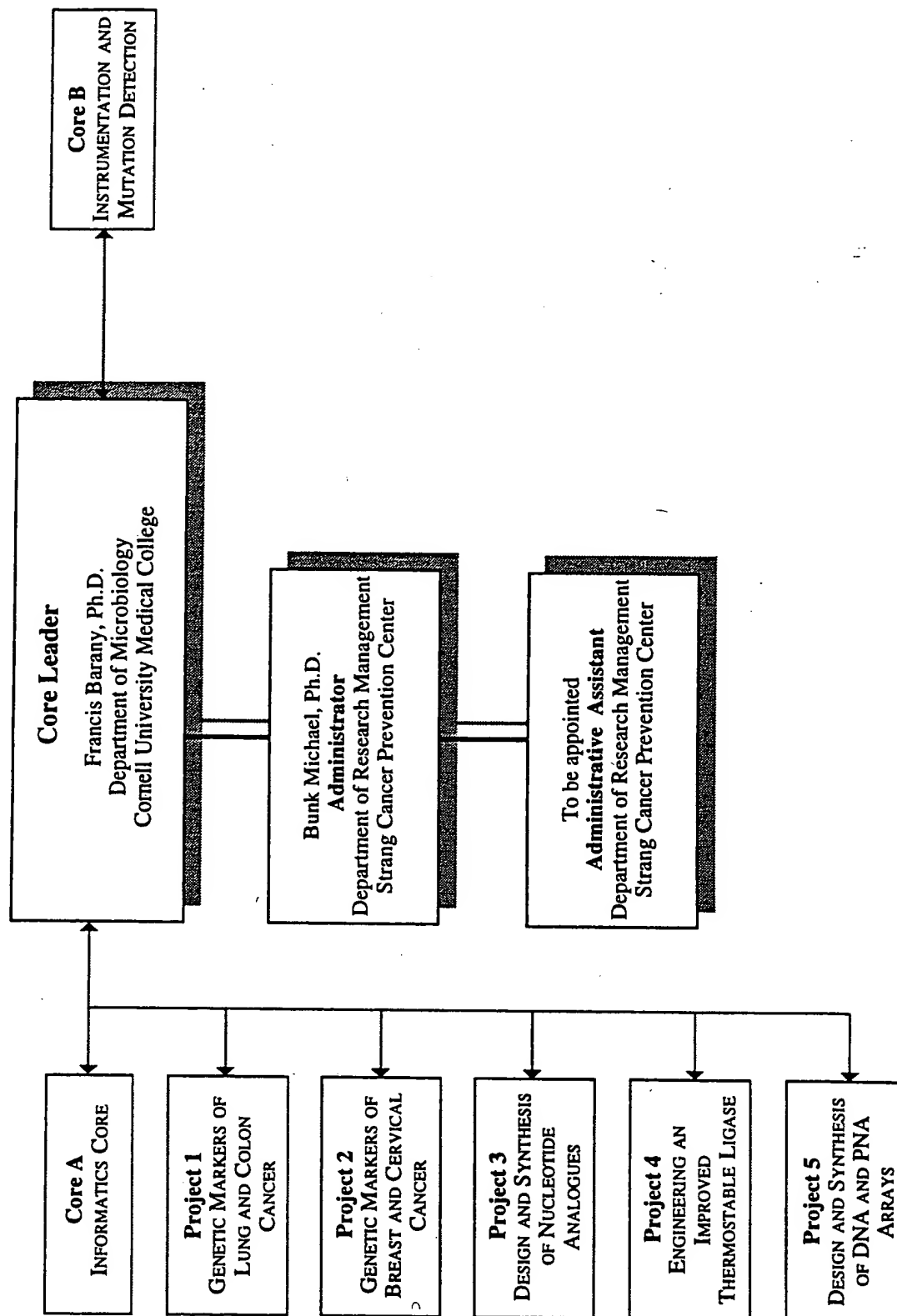


**Project 5 Design and Synthesis of DNA and PNA Arrays**

**Core A Informatics Support For Cancer Detection Methods**





**Core C Administration**

**G. TABLE 2. TABLE OF COLLABORATORS AND LETTERS OF SUPPORT**

Name	Title	Affiliation	Research	Project/Core
Carl Batt	Associate Professor	Cornell University	Detection of <i>Listeria monocytogenes</i> and <i>Erwinia stewardii</i>	Proj. 2, 4, 5 Core A, B
Ron Cook	President	Siris Laboratories	DNA and peptide synthesizers, oligomer arrays	Proj. 3, 5 Core A, B
Jim Coull	Chemistry Group Manager	Millipore Corporation	Peptide-Nucleic Acids PNA arrays Fluorescent detection	Proj. 3, 5 Core A, B
Patrice Courvalin	Professor, Head of Antibacterial Agents Unit	Institut Pasteur	Antibiotic resistance in bacterial pathogens	Proj. 2, 4, 5 Core A, B
Jack Fishman	Professor Director of Research	Cornell University Medical College Strang-Cornell Cancer Research Laboratory	Role of estrogen and estrogen receptor in breast cancer	Proj. 2 Core A, B
Steven Friend	Member	MGH-Harvard Medical College Cancer Center	Detection of p53 mutations in patients with Li-Fraumeni syndrome or with breast cancer	Proj. 1, 2, 5 Core A, B
Wilbur Franklin	Professor	University of Colorado Health Sciences Center	Histologic markers and mutations in soft tissue tumors	Proj. 1
David Gelfand	Director Core Technology	Roche Molecular Systems	Thermostable DNA polymerases, PCR	Proj. 1, 2, 3, 4 Core B
Larry Grossman	Professor & Assoc. Chairman	Wayne State University Sch. of Medicine	Aging and frequency of mutations in mitochondrial DNA	Proj. 1, 2, Core B
Vicky Herrera	Assistant Professor	Boston University Sch. of Medicine	Detection of Na-K-ATPase mutations	Proj. 1, 4 Core B
Eric Hoffman	Assistant Professor	University of Pittsburgh Sch. of Medicine	Detection of hyperkalemic periodic paralysis and other genetic diseases	Proj. 1, 2, 4, 5 Core A, B
Timothy Kennedy	Medical Director Associate Professor	Lung Cancer Inst. of Colorado Univ. of Colorado Health Sciences Ctr.	Advancement of clinical care and early diagnosis of lung cancer.	Proj. 1, Core A, B

Name	Title	Affiliation	Research	Project/Core
Olen Kew	Chief, Molecular Virology Section	Centers for Disease Control and Prevention	Detection of polio virus revertants during vaccine production. Worldwide polio eradication program.	Proj. 1, 2, 3, 4 Core B
John Kovach	Professor & Chairman. Director, NCI designated Mayo Cancer Center	Mayo Clinic	Detection of p53 mutations in patients with breast cancer	Proj. 2, 3, 4, 5 Core A, B
Gary Miller	Associate Professor	Univ. of Colorado Health Sciences Ctr.	Histologic and genetic markers of prostate and colorectal cancer	Proj. 1
Allen Northrup	Principal Engineer	Lawrence Livermore National Laboratory	Development of PCR and LCR amplification on a silicon chip	Core B
Michael Osborne	Professor & Chief Breast Service Director & CEO	New York Hospital-Cornell Medical Ctr. Strang Cancer Prevention Center	Detection of p53 & Her 2/neu & other mutations in patients with Breast Cancer	Proj. 2, 3, 4, 5 Core A, B
David Persing	Assistant Professor	Mayo Clinic	Detection of multidrug resistant <i>Mycobacterium tuberculosis</i>	Proj. 2, 4, 5 Core A, B
Susan Proudfoot	Executive Director	Lung Cancer Inst. of Colorado	Coordination of Lung cancer patient specimens and studies	Proj. 1 Core A, B
Basil Rigas	Associate Professor Acting Division Chief	New York Hospital-Cornell Medical Ctr.	Detection of <i>ras</i> & other mutations in patients with colon cancer	Proj. 1, 2, 4, 5 Core A, B
Richard Roberts	Research Director	New England Biolabs Inc.	Isolation of new thermophilic restriction endonucleases	Proj. 2
Ira Shildkraut	Research Director	New England Biolabs Inc.	Isolation of new thermophilic restriction endonucleases	Proj. 2
Saul Silverstein	Professor & Acting Chair	College of Physicians and Surgeons of Columbia University	Detection of high risk human papillomavirus associated with cervical carcinoma	Proj. 2, 4, 5 Core A, B

Name	Title	Affiliation	Research	Project/Core
John Sninsky	Senior Director of Research	Roche Molecular Systems	Detection of <i>ras</i> & other mutations in patients with pancreatic and other cancers.	Proj. 1, 2, 3 4, 5 Core A, B
Mark Sobel	Chief, Molecular Pathology Section	National Cancer Institute	Detection of HOX gene mutations, and their role in Breast Cancer	Proj. 2, 4, 5 Core A, B
Steven Sommer	Associate Professor	Mayo Clinic	Detection of p53 mutations in patients with breast cancer	Proj. 2, 4, 5 Core A, B
Thierry Soussi	Professor	Institute de Genetique Moleculaire	Detection of p53 mutations in patients with Li-Fraumeni syndrome, breast, lung, gastric, ovary, liver nervous system, colon pancreas cancers and leukemias	Proj. 1, 2, 4, 5 Core A, B
Harold Swerdlow	Research Associate	University of Utah	Development of capillary PCR and LCR amplification and detection	Core B
Perry White	Professor	New York Hospital-Cornell Medical Ctr.	Diagnosis of congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency	Proj. 2, 4, 5 Core A, B
Geoffrey Wilson	Senior staff scientist	New England Biolabs Inc.	Isolation of new thermophilic restriction endonucleases	Proj. 2
Emily Winn-Deen	Staff scientist	Applied Biosystems Division of Perkin Elmer	Multiplex PCR/LDR detection of Cystic Fibrosis and other genetic diseases	Proj. 1, 2, 3, 4 Core A, B



New York State College of Agriculture and Life Sciences  
a Statutory College of the State University of New York

Cornell University

INSTITUTE OF FOOD SCIENCE  
Department of Food Science  
Stocking Hall  
Ithaca, New York 14853-7201  
Telephone 607-255-7616

September 17, 1993

Dr. Francis Barany

Department of Microbiology

Cornell University Medical College

1300 York Avenue, Box 62

New York, NY 10021

Dear Francis:

This letter is to express my interest and enthusiasm for your proposal on "New Methods for Cancer Detection". The success of LCR and more specifically, the productivity of our collaboration is evident in the four peer-reviewed publications, two book contributions and one invited review dealing with LCR that have originated from our joint efforts and carry your laboratory's coauthorship. Our initial efforts to develop a LCR-based system for the detection of pathogenic bacteria focused on *Listeria monocytogenes* as a model system. This pathogen is responsible for over 1,700 known deaths per year in the United States, although this is probably a gross underestimate. Methods for detecting this organism are limited and the utility of a rapid method unquestionable. Our success in formatting this assay coupled to subsequent refinements of this methodology resulting in a non-isotopic, microtiter plate architecture, I believe represent a universal format for other types of assays. As you are aware, we have gone on to develop a similar assay for *Erwinia stewardii*, a plant pathogen. In addition, we have recently completed work on an assay for the genetic disease, bovine leukocyte adhesion deficiency. All of these utilize both PCR to increase sensitivity and LCR to enhance specificity. The coupling of these two powerful technologies has already yielded a highly useful detection strategy and I am confident that your proposed efforts will represent another quantum leap in application of LCR.

We are both in agreement that the next stage is to further increase sensitivity especially in complex target backgrounds. Further, there is a need to interface this *in vitro* enzymatic amplification with the appropriate instrumentation *i.e.* microelectronics. The result will be a fully integrated technology taking these molecular biological tools into the realm, now routinely available for simple metabolite analysis, as exemplified by glucose analysis. I therefore share your interest and vision and look forward to our continuing collaboration.

Sincerely yours,

Carl A. Batt

Associate Professor

(607)255-2896

FAX (607) 255-8741

BAT@CORNELLA.BITNET

# SIRIS Laboratories

40 Mark Drive  
San Rafael, Ca 94903  
Tel 415-479-8710, Fax 479-1606

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September 16, 1993

Dr. Francis Barany  
Dept of Microbiology, Box 62  
Cornell University Medical College  
1300 York Ave.  
New York, NY 10021

Dear Francis,

I write to express my enthusiastic interest in helping you with the research program on "New Methods for Cancer Detection". As Founder of Biosearch (1977), I have extensive experience in the development and commercialization of novel chemistries and instrumentation for biotechnology. As Chief Scientific Officer of Biosearch, I was responsible for all product development which included DNA and peptide synthesizers and associated chemistries, as well as protein and DNA sequencing. More recently, I have founded Siris Laboratories which will develop technology for the generation and application of biopolymer arrays. We believe arrays will provide an enabling foundation for the development of advanced methods for drug discovery as well as diagnostic medicine. From this background, I am in a position to understand the significance of your work, and to contribute to solving the technical issues that will arise in producing simple, reliable, and inexpensive oligonucleotide or PNA arrays for clinical use.

Recently, I have developed concepts for the practical preparation of biopolymer arrays using standard chemistries. This process which is now in the process of being reduced to practice, will allow the facile preparation of moderately dense arrays (<1000 elements per square cm). Such arrays should be readily integrated with your concept for the LDR mediated detection of cancer and genetic disease.

My colleague Dr. Derek Hudson and I have interacted with your co-investigator Dr. George Barany since the mid-1980's, and this collaboration has led to a number of reagents and support materials for solid phase synthesis of peptides. We are confident of our ability to achieve similar advances for array based DNA and PNA molecules.

With best wishes for the success of your program.

Sincerely yours,



Ronald M. Cook, Ph.D.  
President

# MILLIPORE

September 12, 1993

Dr. Francis Barany  
Dept. of Microbiology, Box 62  
Cornell University Medical College  
1300 York Avenue  
New York, NY 10021  
Tel. 212-746-6509

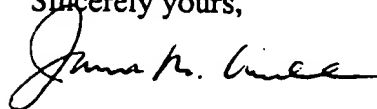
Dear Francis,

On behalf of Millipore Corporation and myself, I write to indicate our willingness to help in any way possible the research described in your application to the National Institutes of Health/National Cancer Institute entitled "New Methods for Cancer Detection". I should add that your co-investigator Dr. George Barany is a long-time consultant for Millipore, and the academic-industrial collaboration between his laboratory and our company has led to several highly useful and commercially successful handles, amino acid derivatives, and polymeric supports for solid-phase peptide synthesis.

Millipore holds an exclusive license on the synthesis of Peptide-Nucleic Acids (PNA). We have an extensive collaboration with the Copenhagen-based inventors of PNA, Ole Buchardt, Peter Neilsen, Michael Egholm and Rolf Berg as well as scientists at Isis Pharmaceuticals and PNA Diagnostics. It has now been demonstrated that PNA/DNA hybrids are considerably more stable than the corresponding double stranded DNA structures. Furthermore, PNAs bind to nucleic acids with greater specificity (i.e. more discrimination) than their natural DNA and RNA counterparts. Millipore has contributed greatly to advances in the chemistry of PNA synthesis and it is now possible to routinely prepare by automated means PNAs of <20 residues containing the four natural nucleobases. My group has also developed methods for labelling of PNAs as well as linkers for the chemical attachment of PNA to solid supports, including proprietary low-fluorescence membranes produced by Millipore.

Millipore can help in your efforts to create PNA arrays for fluorescent detection of diseases. We are in a position to supply you with all necessary reagents and state-of-the-art synthesis protocols for PNA. I understand that all of these technologies may play a vital part in the implementation of your ideas, and am very much looking forward to helping you with your important studies.

Sincerely yours,



James M. Coull, Ph.D.  
Specialty Chemistry Group Manager

cc: Jack Johansen  
Sr. V.P. Science and Technology

## Institut Pasteur

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Dr. F. BARANY

Department of Microbiology

Box 62

Cornell University Medical College

1300 York Avenue

NEW YORK, NY 10021

(USA)

PC/mo

Paris, September 29th, 1993

Dear Francis,

I am writing this letter of support for your grant application to the National Cancer Institute entitled "New Methods for Cancer Detection". This is a very creative idea, which holds significant promise not only for treatment of cancers, but also for therapy of infectious diseases based on detection of antibiotic resistance.

I am very enthusiastic about collaborating with you on multiplex PCR/LDR technology. This has immediate use for the early detection and identification of antibiotic resistant pathogenic microbes. Recently, my laboratory has demonstrated that "new" extended-spectrum  $\beta$ -lactamases in *Enterobacteriaceae* are point mutants of "old" penicillinases which allows their detection and identification by "oligotyping". We have also elucidated both the genetics and biochemistry of glycopeptide resistance in *Enterococcus* spp. In addition, we have characterized, in Gram-positive cocci and Gram-negative bacilli, chromosomal genes that are responsible for low-level, species specific, resistance to antibiotics. Probes or oligonucleotides complementary to these genes allow both detection of resistance and precise identification of the host. By working together, we could design the appropriate primers for rapid multiplex identification of several pathogens, as well as determining if they carry antibiotic resistance genes.

The emergence of  $\beta$ -lactamase mutant strains of *Klebsiella pneumoniae*, *Escherichia coli*, *Salmonella* spp. etc., has considerably limited the number of antibiotics left for the treatment of severe systemic infections due to these microorganisms since the strains are resistant not only to all penicillins but also to monobactams and all cephalosporins with the exception of cephamycins (however, use of this latter class of antibiotics readily selects porin mutants !). Emergence of vancomycin resistance in *Enterococcus* is also worrying since this genus is already multiply resistant to antibiotics. As you may know, the enterococci responsible for a current epidemic of nosocomial infections in Cleveland-Ohio are resistant to all antibiotics and the death rate of infected patients is 100 %.

As detailed in my 1991 review (Antimicrob. Agents Chemother. 35:1019-1023), the genotypic approach to the study of bacterial resistance to antibiotics is most adapted to: 1) Infections due to slowly growing bacteria such as *Mycobacterium* spp.; the fastidious bacteria



anaerobes, capnophilic Gram-negative bacilli, etc.; the *in vivo* cultivable bacteria *Rickettsia* and *Treponema* spp.; and the "dangerous" bacteria *Brucella* spp. *Francisella tularensis*, and *Mycobacterium tuberculosis*; 2) Therapeutic emergencies with antibacterial agents (*i.e.*, meningitis in a patient allergic to  $\beta$ -lactams, neonatal meningitis, septic shock, septicemia due to Gram-negative bacteria, and staphylococcal infections); 3) Resistance mechanisms difficult to detect (*e.g.*, low-level glycopeptide resistance in *Enterococcus* spp.); and 4) The numerous pitfalls in phenotypic detection.

The indiscriminant use of antibiotics has made multiresistant pathogens an international problem, and we must prepare ourselves for the "post antibiotic era" in medical microbiology. The methods you are developing is our first line of defense.

Sincerely yours,



P. COURVALIN



# STRANG-CORNELL CANCER RESEARCH LABORATORY



Joint Program of the Strang Cancer Prevention Center and Cornell University Medical College

September 24, 1993

Francis Barany, Ph.D.  
Associate Professor of Microbiology  
Cornell University Medical College  
1300 York Avenue  
New York, NY 10021

Dear Francis:

I am extremely impressed with your development of the ligase chain reaction as a method of mutation detection. Its application to cancer diagnosis and to the resolution of the progressive steps involved in the carcinogenic process opens truly new horizons. I would be most interested in collaborating with you in your program project "New Methods for Cancer Detection." In particular we wish to apply your methodology to the identification of the genetic lesions induced by the covalent binding of the endogenous estrogen 16 $\alpha$ -hydroxyestrone to the estrogen receptor. We have ample physiological and epidemiological evidence for the role of this reaction in the induction and/or promotion of breast and endometrial cancer. The use of the ligase chain detection method in our cell and tissue culture studies as well as the ongoing in vivo studies can well shed new light on the mechanism of hormonal carcinogenesis.

I am looking forward to an extremely fruitful collaboration.

Sincerely,

Jack Fishman, Ph.D.  
Professor, Cornell University Medical College  
Director of Research  
Strang-Cornell Cancer Research Laboratory

University of Colorado Cancer Center  
Lung Cancer SPORE Program

Campus Box B188  
4200 East Ninth Avenue  
Denver, Colorado 80262  
(303) 270-7167  
FAX: (303) 270-3304

January 4, 1994

Vincent L. Wilson, Ph.D.  
Director, Molecular Genetic/Oncology  
Department of Pathology, B-120  
The Children's Hospital  
1056 E. 19th Ave.  
Denver, CO 80218

Dear Vince:

We are most anxious to assist you in your search for early and intermediate markers of malignant transformation in bronchial epithelium through the application of sensitive methods for the detection and identification of point mutations. We have already made considerable progress in our efforts to provide you with specimens for your preliminary studies and I anticipate that we will be able not only to continue these efforts but will also be able to fine tune the specimen preparation and storage to fit your laboratory's needs.

For your proposed project on oncogenic mutational markers in lung, the Colorado Lung SPORE Tissue Bank Core laboratory will provide you with tumor, adjacent normal lung and peripheral blood DNA from resections of invasive tumors and dysplastic epithelial lesions from both resection specimens and bronchial biopsies. Clinical and histological data will be available on all specimens studied. We estimate that tumor, non-invasive bronchial mucosa and peripheral blood will be available from approximately 75 patients per year and bronchial biopsy material and peripheral blood from about 200 patients per year. At present the core laboratory is storing more than 50 resected lung cancers of all types with uninvolved lung tissue and peripheral blood and more than 150 bronchial biopsy specimens with peripheral blood. In addition, we have begun to analyze p53 mutations in our tumors by SSCP and DNA sequencing of PCR products and our sequence data will be available for comparison with your analysis of sputum and biopsy specimens from these same patients.

Good luck with your proposal. If I can be of further help, please let me know.

Sincerely,



Wilbur A. Franklin, MD  
Director, Tissue Bank Core Laboratory

MASSACHUSETTS GENERAL HOSPITAL — HARVARD MEDICAL SCHOOL  
THE MGH CANCER CENTER

Building 149, 13th Street  
Charlestown, MA 02129  
Telephone (617) 726-5600

September 28, 1993

Dr. Francis Barany  
Associate Professor of Microbiology  
Cornell University Medical College  
1300 York Avenue, Box 62  
New York, N.Y. 10021

Dear Francis:

I am glad to hear of your efforts for new methods for cancer detection. If you think it will help to have access to our information regarding germline p53 mutations we will be delighted to provide that to you.

I have enclosed a list of germline p53 mutations and references. I also have included our paper coming out in Nature Genetics which takes a different approach that considers functional inactivation.

I think I realize how much effort goes into organizing these program project grants and wish you the best of luck.

Sincerely,



Stephen H. Friend, M.D., Ph.D.  
Member, MGH Cancer Center

Encls.

Sent by Fedex



## Roche Molecular Systems

a subsidiary of Hoffmann-La Roche

Roche Molecular Systems, Inc.  
1145 Atlantic Avenue  
Alameda, CA 94501  
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TEL: (510) 814-2853  
FAX: (510) 814-2997

September 24, 1993

Dr. Francis Barany  
Department of Microbiology, Box 62  
Cornell University Medical College  
1300 York Avenue  
New York, NY 10021  
TEL: (212) 746-6509

Dear Francis:

We are writing in support of your grant application to the National Institutes of Health/National Cancer Institute entitled "New Methods for Cancer Detection". You have developed an innovative concept and set of approaches which hold promise for detection, prognosis and treatment of cancers.

Our own research efforts and those of our colleagues at Cetus, and now Roche Molecular Systems, led to the development of many of the advances in PCR amplification; representative examples include: cloning thermostable *Taq* polymerase, *Tth* polymerase, *Tma* polymerase, quantitative PCR, enhanced specificity, carryover control and greater single base discrimination. We have closely followed your LCR work, and one of us (D.G.) has enjoyed a fruitful collaboration which has already led to a joint publication (Barany, F. and Gelfand, D. (1991) *Gene* 109:1-11).

We can assist your efforts in a number of ways. We have and shall continue to share our experience in PCR amplification and disease detection. Different thermostable polymerases will be made available to you for testing the fidelity of extension using your various nucleotide analogs. We are also currently providing financial support to your lab for cloning of additional thermostable DNA replication accessory proteins. This Roche-sponsored work does not overlap with your cancer detection work, but may lead to improvements in the speed and specificity of DNA amplification in general.

We are keenly interested in following the progress of your program project grant. Good luck in attracting the funds for the program project.

Sincerely yours,



David Gelfand, Ph.D.  
Director, Core Technology



John Sninsky, Ph.D.  
Senior Director, Research



WAYNE STATE UNIVERSITY SCHOOL OF MEDICINE

DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

Lawrence I. Grossman, Ph.D.  
Professor and Associate Chairman

Scott Hall of Basic Medical Sciences  
540 East Canfield Avenue  
Detroit, Michigan 48201

(313) 577-5326 • (313) 577-5218 fax  
Lgrossman@mts.cc.wayne.edu

1 September 1993

Dr. Francis Barany  
Department of Microbiology  
Cornell University Medical College  
1300 York Avenue  
New York, NY 10021

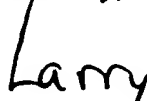
Dear Francis:

I was excited to hear of the advances you have made in using the ligase chain reaction both to detect rare mutations and to measure their frequency. As you know, I am interested in mitochondrial DNA mutations, particularly in their accumulation with age. Some cutting edge questions are: What is an accurate estimate of the frequency of any particular mutation? What is the total frequency of mutations? Are deleterious mutations selectively amplified? These questions are important because they bear on the issue of whether the accumulation of mutations with age is causally related to aging or perhaps just represents thermodynamic noise.

The mutations of interest are in the range of  $10^{-3}$  to  $10^{-6}$ . Current methodologies fall short of being able to accurately measure this frequency of mutation, and I believe the ligase chain reaction holds great promise in this regard.

Consequently, I would be pleased to collaborate with you on applying your technology to these and other important questions that may be of mutual interest, and look forward to keeping in touch with new advances as they become available.

Sincerely,

  
Lawrence I. Grossman

LIG:ms

Principal Investigator/Program Director: BARANY, FRANCIS



Boston University  
School of Medicine

Section of  
Molecular Genetics  
Cardiovascular Institute  
Department of Medicine  
Housman Medical  
Research Center  
80 East Concord Street  
Boston, Massachusetts  
02118-2394  
TEL: 617 638-5077 or 5085  
FAX: 617 638-5141

*Faculty*  
Vassilis I. Zannis, Ph.D.  
Professor, Director  
Nelson Ruiz-Opazo, Ph.D.  
Associate Professor  
Christos Cladaras, Ph.D.  
Assistant Research Professor  
Victoria L.M. Herrera, M.D.  
Assistant Professor  
Alex Mitsialis  
Assistant Research Professor  
Margarita Hadzopoulou-  
Cladaras, Ph.D.  
Research Instructor  
Eleni E. Zanni, Ph.D.  
Research Instructor

September 29, 1993

Dr. Francis Barany  
Department of Microbiology Box 62  
Cornell University Medical College  
1300 York Avenue  
New York, New York 10021

Dear Dr. Barany:

We are writing to officially document our objective scientific assessment as to the importance of the isolation and characterization of the thermostable ligase enzyme and its application to ligase chain reaction (LCR) genetic analysis. Our assessment is based on direct experience with the use of the LCR technique as a necessary corroborative experimental test to clarify whether a mutation exists in the  $\alpha 1$  Na,K-ATPase gene in the Dahl salt-sensitive hypertensive rat strain. In collaboration with you, the LCR technique provided critical corroborative evidence of the presence of the T<sup>1079</sup> mutation in the  $\alpha 1$  Na,K-ATPase gene. Its value is emphasized in the fact that it provides another enzymatic genetic assay critical to independent confirmation of mutations identified by PCR amplification techniques using the Taq polymerase.

Most importantly, we are very grateful for your willingness to teach and collaborate with us on performing the LCR analysis accurately and expeditiously. We look forward to your continued scientific leadership in the field as well as your continued success. Its important to all of us.

Sincerely,

*Nelson Ruiz-Opazo*  
Nelson Ruiz-Opazo, Ph.D.

*Victoria L.M. Herrera*  
Victoria L. M. Herrera, M.D.



# University of Pittsburgh

SCHOOL OF MEDICINE  
Department of Molecular Genetics  
and Biochemistry  
Biomedical Science Tower, Room E1240

September 12, 1993

Francis Barany, PhD  
Associate Professor of Microbiology  
Cornell University Medical College  
1300 York Ave.  
New York, NY 10021

Dear Francis,

This letter is in reference to your program project application entitled "New methods in cancer detection" to be submitted to the NIH.

First, let me express my enthusiasm for continuing collaboration on applications of LCR for mutation detection in genetic neurological disease. The extension of your techniques towards multiplexing and miniturization, and their potential application to our collaborative work on periodic paralysis and Duchenne muscular dystrophy are particularly exciting. Our collaborative work has led to two joint publications in little over 1 year, and I anticipate that our future work will be equally productive.

My laboratory is focused on determining the molecular basis for a number of neuromuscular diseases, and directly applying this knowledge to molecular diagnostics and therapeutics. Diseases currently under active investigation are Duchenne/Becker muscular dystrophy (one of the most common and most devastating human inherited diseases), periodic paralysis, paramyotonia congenita, rippling muscle disease, Becker's myotonia, Thomsen's myotonia, spastic paraparesis, and congenital myasthenia gravis. One of our current RO1 grants is to automate genetic linkage analysis using automated sequencers and artificial intelligence, and this work may dovetail nicely with your planned research.

Using LCR, we have been able to multiplex the detection of three distinct sodium channel mutations, and their corresponding normal alleles (6 sites total) in a single reaction, with detection on automated sequencers. We are currently expanding this to detect about 20 sites using multiple fluors for detection. The computer algorithms we have developed in collaboration with Mark Perlin of Carnegie-Mellon computer science department for genotyping of CA repeat alleles on automated sequencers should be directly applicable to large-scale multiplexing of LCR products.

I wish you success with your important proposal, the described experiments will certainly have a positive influence on our work on neuromuscular disease.

Best regards,

A handwritten signature in black ink, appearing to read "Eric P. Hoffman".

Eric P. Hoffman, PhD





L U N G C A N C E R  
INSTITUTE OF COLORADO

January 4, 1994

Vincent L. Wilson, Ph.D.  
Director, Molecular Genetics/Oncology  
Department of Pathology, B-120  
The Children's Hospital  
1056 E. 19th Avenue  
Denver, CO 80218

Dear Vince:

We enjoyed our discussion regarding your planned grant application concerning the identification of oncogenic point mutations as early and intermediate markers of lung cancer. As we have discussed earlier, we will be more than pleased to continue the collaborations we have recently established under the University of Colorado Cancer Center's "Specialized Program of Research Excellence (SPORE)" pilot grant that you received. We believe that your work has some fascinating possibilities and we look forward to these studies.

As the Directors of the Tissue Procurement CORE II under the SPORE, we can provide you with sputum, bronchoscopy mucosal brushings and biopsies, and peripheral blood from subjects we are enrolling into our regional sputum screening system. As you are aware, the Lung Cancer Institute of Colorado has established a consortium of community and institutionally-based pulmonologists and family practitioners which extends throughout the Greater Rocky Mountain Region. Through this consortium, we are accruing approximately 50 patients (at high risk for lung cancer) per month and have already successfully procured various types of specimens from 220 subjects to date. We estimate that approximately 210 subjects with mild dysplasia, 100 subjects with moderate to severe dysplasia, and 20 subjects with carcinoma *in situ* will be cytologically diagnosed annually for the remaining two years of the SPORE. It is also anticipated that in 10-15 cases per annum, sputum, bronchoscopy biopsy, and blood will be collected from patients where lung tumor tissue is also available.

Good luck with your proposal. If we can be of further help, please let us know.

Sincerely,

Timothy C. Kennedy, M.D.  
Medical Director  
(303) 863-0300

Susan P. Proudfoot, M.S.H.A.  
Executive Director  
(303) 778-7023

TCK/SPP:dw

Centers for Disease Control  
and Prevention (CDC)  
Atlanta GA 30333

24 September 1993

Francis Barany, Ph.D.  
Associate Professor of Microbiology  
Cornell University Medical College  
1300 York Avenue, Box 62  
New York, N.Y. 10021

Dear Dr. Barany:

We welcome continuation of our collaboration on the applications of your technologies for detecting small proportions of allelic variants in genomic populations. Several important problems in virology can be better approached through the application of LCR/PCR and the newer methods under development in your laboratory. Among these is the problem of vaccine quality control.

My laboratory is responsible for providing virologic support for the World Health Organization's Global Poliomyelitis Eradication Initiative. We have served since 1985 as the primary reference laboratory for the successful Poliomyelitis Eradication Initiative in the American Region. The worldwide incidence of paralytic poliomyelitis is currently estimated to be 120,000/yr, primarily striking children <2 years of age. The goal is to eradicate poliomyelitis by the year 2000. A major activity of my laboratory is to develop highly sensitive and reliable methods to detect wild poliovirus circulation, and, whenever feasible, to transfer these technologies to other laboratories within a global network supporting the Initiative. We have developed reagents and methods based upon PCR and probe hybridization for the rapid identification of polioviruses. Virologic surveillance is an essential supplement to standard epidemiologic surveillance because: 1) other viral infections can mimic poliomyelitis (> 1000 paralytic cases still occur in the Americas), but none are associated with wild poliovirus infections, 2) vaccine-associated poliomyelitis occurs in about 1 in 500,000 vaccinees or contacts (~4-6 vaccine-associated cases/yr occur in the United States), and 3) the large majority (>99%) of wild poliovirus infections are subclinical. Thus, at the final stages of poliomyelitis control it is necessary to sample healthy children and the environment for evidence of wild poliovirus circulation. Our continued collaboration will facilitate development of improved reagents and methods.

A severe impediment to global polio eradication is the availability of sufficient oral poliovaccine to meet worldwide demand. The current methods of poliovaccine production in primary monkey kidney cells are comparatively slow and expensive. If vaccines could be prepared at large scales (e.g., in bioreactors) in alternative cell substrates (such as continuous cell lines), the problem of poliovaccine cost and availability may be surmounted. A difficulty associated with poliovaccine production in any system is the selection in bulk culture of variants having

Dr. Francis Barany (24 Sept 93): page 2

increased neurovirulence. Back-mutation (to wild type) at a single nucleotide position for each of the attenuated poliovaccine strains (there are three poliovirus serotypes) is largely responsible for this phenotypic reversion. We and others have developed methods capable of detecting revertant genomes at a sensitivity of 1 in  $10^3$  or  $10^4$ . However, the greater sensitivity and specificity of PCR/LDR is required to measure the kinetics of variant selection in culture to be monitored with high precision, opening the way for developing improved conditions for poliovaccine production. The applications of such technology to viral vaccinology and diagnostics extend well beyond this specific example.

An emerging problem in virology is the appearance of mutants resistant to antiviral drugs. This is clearly a major problem for the treatment of HIV-infected patients, but the problem is much broader. Sensitive methods are needed to follow the dynamics of selection of drug-resistance mutations, particularly to assess the impact of combined drug therapies. Many fundamental questions of drug and antibiotic resistance can be approached through the PCR/LDR methods you are developing. These approaches also have potentially important implications for diagnostics, patient management, and epidemiology.

I look forward with enthusiasm to our continued collaboration.

Sincerely yours,



Olen Kew, Ph.D.  
Chief, Molecular Virology Section  
Respiratory & Enteric Viruses Branch  
Division of Viral & Rickettsial Diseases  
National Center for Infectious Diseases  
Centers for Disease Control & Prevention  
Atlanta, GA 30333 USA  
FAX: 404-639-1307

Mayo Clinic

Rochester, Minnesota 55905 Telephone 507 284-2511

Comprehensive Cancer Center

November 24, 1993

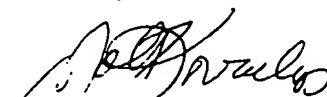
Dr. Francis Barany  
Associate Professor of Microbiology  
Cornell University Medical College  
Department of Microbiology  
1300 York Avenue, Box 62  
New York, NY 10021

Dear Doctor Barany:

My colleague and collaborator, Dr. Steve S. Sommer, and I would be pleased to provide you with a printout of our compendium of p53 mutations in human cancers. We are also able to provide you with amplified segments of the p53 genome from human breast cancers which contain mutations. In most instances, we also can provide wild-type DNA from uninvolved lymph nodes of the same individual. We have approximately 75 different p53 mutations. Among those are all classes of mutations.

We are very interested in your attempts to develop more powerful methods for the detection of mutations. Improved methodology would be highly advantageous to our molecular epidemiological approaches to breast cancer.

Sincerely,



John S. Kovach, M.D.  
Professor and Chair  
Department of Oncology  
Director, NCI-Designated Mayo  
Comprehensive Cancer Center

JSK:bp



Department of Pathology

Campus Box B216  
4200 East Ninth Avenue  
Denver, Colorado 80262  
(303) 270-6721 FAX  
(303) 270-8171  
(303) 270-7636

School of Medicine

January 10, 1994

Vincent L Wilson PhD  
Director, Mol Genet/Oncol  
Department of Pathology B120  
The Children's Hospital  
1056 E 19th Avenue  
Denver CO 80218

Dear Vince:

Your proposal of studying point mutations as early and intermediate markers of colon cancer sound very exciting. The ability to detect and identify oncogenic point mutations at sensitivities of greater than one in a million cells, as your techniques suggest, may provide the opportunity to detect colorectal cancer at an early stage.

As a pathologist and the Director of the Histopathology/Tissue Procurement Core Laboratory of the University of Colorado Cancer Center, I would be more than happy to collaborate on this project. I can provide you with tumor and adjacent normal tissue (where available). Clinical and histological data will be available on all specimens studied. It is estimated that approximately 12-15 tumor and adjacent normal tissue specimens can be provided per year.

Good luck with your proposal. If I can be of further help, please let me know.

Sincerely,

Gary J. Miller, M.D., Ph.D.  
Professor

cm



## Lawrence Livermore National Laboratory

September 28, 1993

Dr. Francis Barany  
Dept. of Microbiology, Box 62  
Cornell University Medical College  
1300 York Ave.  
New York, NY 10021  
Tel.# 212-746-6509

Dear Francis,

I am writing a letter of collaboration for your grant application to the National Institutes of Health/National Cancer Institute entitled "New Methods for Cancer Detection". I have read your visionary cancer detection proposal and believe that your approach will usher in a new era of cancer care through early diagnosis and preventive treatment.

As you know, my laboratory has recently developed a miniaturized thermal cycling device for PCR amplification. The reaction chamber is etched in silicon, with an integrated polysilicon thin-film heater and feedback temperature control. We have achieved successful PCR amplifications at rates 4 times faster than is possible with current commercial instruments. The power level required for this microinstrument is on the order of a fraction of a watt, allowing for potential battery operation. We are currently in the process of developing a more complete DNA analysis microinstrument consisting of integrated reaction chambers, fluidic devices, and detection methodology. This type of miniaturized, integrated, instrument will benefit the ultimate utility of clinical diagnostic tools such as the methodology you are developing.

We are currently improving our designs and building micromachined PCR reaction chambers and related structures. Since LCR and LDR work by the same thermal cycling principles as PCR, these chambers are immediately applicable to your work. Furthermore, we are developing serial reaction chambers for nested PCR and miniaturized diagnostic instruments. Such designs could automatically add reagents under hot start conditions, and dilute or move products into subsequent reaction or detection chambers. Since these devices will be self-contained, they will also solve PCR "carryover" problems. These chambers are immediately applicable to your cancer detection strategies and we will work together in the future to test these strategies in micro-device based instruments. Your access to this technology will be worked out within the structure of our present industrial collaborations.



My laboratory has just received significant funding from ARPA, and is about to receive additional support in a collaborative effort from Perkin Elmer/ABI and Roche Molecular Systems. Thus, your cancer detection program completely complements our microfabrication program, and we are working with the same industrial partners. In view of the current funding constraints faced by academic researchers, it is perhaps best that your NCI/NIH funding be used solely for the support of academic researchers. As a government employee, with substantial government and industrial support, our funding level is currently sufficient for the type of microinstrument you are interested in. Should a need for additional funding arise in the future, we could pursue it collaboratively.

I am excited about working with you on your cancer and disease detection projects and believe your work will have a fundamental impact on health care in this country.

Sincerely yours,



M. Allen Northrup, Ph.D.  
Principal Engineer  
Engineering Research Division, L-222

## THE NEW YORK HOSPITAL-CORNELL MEDICAL CENTER

MICHAEL P. OSBORNE, M.D., F.A.C.S.  
PROFESSOR OF SURGERY  
CHIEF, BREAST SERVICE  
DEPARTMENT OF SURGERY

PATIENT APPOINTMENTS  
(212) 794-6085  
ADMINISTRATION  
(212) 746-6629

November 23, 1993

Dr. Francis Barany  
Department of Microbiology  
Cornell University Medical College  
1300 York Avenue  
New York, NY 10021

Dear Francis:

I am pleased to be able to confirm the full collaboration of the Breast Service at The New York Hospital-Cornell Medical Center in your research efforts. Your very exciting work on the potential applications of combined PCR and LDR hold enormous promise for advancing breast cancer research and treatment. Of great importance is the correlation of mutations in oncogenes and the evolution and prognosis of the disease. My particular interest in your technology relates to the detection of micrometastases in patients with breast cancer. I have carried out studies in over 600 women using monoclonal antibody and there is substantial room for improvement in this technology.

The full resources of the Strang Cancer Prevention Center and the Strang-Cornell Research Laboratory are behind your efforts. We have in house several breast cancer cell lines well characterized with respect to gene amplifications and p53 mutations. In addition, similarly characterized cell lines are available through my collaborators. Other specimens which will be available for your investigations include frozen primary breast tumor specimens, lymph nodes, fixed tissue specimens, and the above mentioned bone marrow specimens from the same patients. These samples will be from patients whose course of disease has been followed for several years, ultimately allowing for correlation of genetic mutations with clinical outcome.

In addition, we have access to bone marrow samples from patients with active breast cancer and those in remission whose primary tumor and lymph node tissue samples you will have already characterized. Such samples would be ideal for your powerful new method of identifying one cancer cell from a background of million of normal cells.

Finally the potential utility of PCR and LDR in screening family members of breast cancer patients and other populations at risk are tantalizing. We look forward to an exciting collaboration with your laboratory.

Yours sincerely,



Michael P. Osborne, MD

/tp





## Mayo Clinic

200 First Street Southwest Rochester, Minnesota 55905 Telephone 507 284-2511

David H. Persing, M.D., Ph.D.  
*Experimental Pathology and Laboratory Medicine*

September 9, 1993

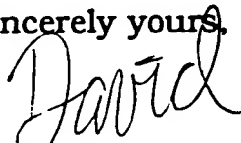
Dr. Francis Barany  
Department of Microbiology  
Box 62  
Cornell University Medical Center  
1300 York Avenue  
New York, NY 10021

Dear Francis:

This letter is in support of your project on the use of ligase chain reaction and ligase detection for the analysis of single base mutations. As you know, we have been interested in developing rapid detection methods for multidrug resistant tuberculosis, and have focused on detection of mutations within *rpoB*, the gene encoding rifamin resistance in *Mycobacterium tuberculosis*.

Because of the variety of mutations encountered within *rpoB*, we are very intrigued by the possibility of using ligation mediated detection for simultaneous detection of multiple mutations within an amplified locus. The technique you are investigating will undoubtedly prove to be valuable for this kind of analysis, as well as for other infectious disease genetic targets.

Sincerely yours,



David H. Persing, M.D., Ph.D.

DHP:djh

## THE NEW YORK HOSPITAL-CORNELL MEDICAL CENTER

DEPARTMENT OF MEDICINE  
DIVISION OF DIGESTIVE DISEASES  
TEL. (212) 746-4400  
FAX (212) 746-8447

September 17, 1993

Dr. Francis Barany  
Associate Professor of Microbiology  
Cornell University Medical College  
1300 York Avenue  
New York, NY 10021

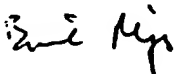
Dear Francis:

I am writing to confirm that the Division of Digestive Diseases and my own laboratory will be happy to collaborate with you fully on the applications of the ligase chain reaction (LCR) to colon cancer.

There are several critical questions on the biology of colon cancer with respect to its evolution and metastasis that can best be evaluated by your novel and powerful methodology. In fact, as we have discussed, there are aspects of colon cancer that can be assessed only by your approach. This explains our enthusiasm about this collaborative effort and our excited anticipation of very important results.

As you know, my laboratory has had extensive experience with both the development of diagnostic methodology for colon cancer (infrared spectroscopy) and the study of colon cancer immunology (HLA gene regulation). In addition, in our inpatient and outpatient clinical services we are following a large number of patients with colon cancer, several patients with familial adenomatous polyposis and a large number of patients with sporadic colon polyps. This patient population, which is clinically and pathologically studied in detail, will provide the clinical background for these studies. In planning our collaboration, we have obtained approval by our Human Investigations Committee to study tissues by LCR.

Sincerely yours,



Basil Rigas, M.D.  
Associate Professor of Medicine  
and Microbiology  
Acting Chief, Division of Digestive Diseases





NEW ENGLAND

**Biolabs**

32 Tozer Road Beverly, MA, U.S.A. 01915

(508) 927-5054  
TELEX 6817316  
FAX (508) 921-1350

September 23, 1993

Dr. Francis Barany  
Cornell University Medical College  
Department of Microbiology  
1300 York Avenue  
Box 62  
New York, NY 10021

Dear Francis:

It was a pleasure finally to hear the details of your innovative method for detecting mutations. Obviously, it falls right in the middle of one area of our research here at New England Biolabs and we would welcome the opportunity to collaborate with you on developing the enzymological aspects of it.

As you know we have had a major commitment in our research effort to isolate and characterize new restriction endonucleases. We were pioneers in the cloning and sequencing of these systems and currently have more than 50 such systems cloned. We have devoted much attention to the crystallization of restriction enzymes and methylases and have recently been successful in obtaining structures for the *Bam*HI restriction endonuclease in collaboration with Dr. Aneel Aggarwal, and the *Hha*I DNA methyltransferase in collaboration with Dr. Xiaodong Cheng. Obviously, these structural studies will be of great benefit in our future research efforts to engineer desirable properties into these proteins.

For some time now, we have been screening thermophilic organisms for proteins that would be of use as reagents for molecular biology research. We already have a number of thermophilic restriction enzymes and are constantly seeking more. We also have thermostable DNA polymerases including two which we are already selling, Vent and Deep Vent polymerases (with and without 3'-5'proofreading activities). Both the polymerases and the thermophilic restriction enzymes might be of use in your project. We will supply you with samples of any of our new thermophilic restriction enzymes or DNA polymerases, that are not available through our catalog, so that you might test them in your cancer detection schemes. We had already considered stepping up our screening efforts on thermophilic

organisms and your project would be a strong stimulus for us to move these plans forward. Furthermore, because of the specific nature of the enzymes that would be useful to you, we might hope to design screens that would help to identify suitable enzymes quickly.

We are happy to hear that there is interest at the National Cancer Institute in funding your work and wish you success in your application. We are excited that we might be able to make a significant contribution to your work. Working with you over the past seven years on enzymes from *Thermus aquaticus* has been rewarding and we look forward to a productive and intellectually exciting collaboration in the future.

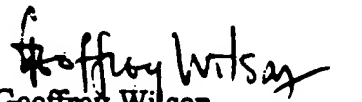
Yours Sincerely,



Richard J. Roberts  
Research Director,



Ira Schildkraut  
Research Director,



Geoffrey Wilson  
Senior Staff Scientist,

TEL: (508) 927-3382  
FAX: (508) 921-1527  
E-MAIL: roberts@neb.com

RJR:ll

College of Physicians & Surgeons of Columbia University | New York, N.Y. 10032

DEPARTMENT OF MICROBIOLOGY  
Saul J. Silverstein

701 West 168th Street  
(212) 305-8149  
Fax (212) 305-1468

9/21/93

Francis Barany, PhD  
Dept. of Microbiology  
Cornell University Medical College  
1300 York Ave. Box 62  
New York, N.Y. 10021

Dear Francis:

I am writing to express my willingness to collaborate with you on the Program Project Grant Application entitled "New Methods for Cancer Detection" that you are submitting to the NCI. As always, I look forward to the opportunity to share our ideas with you in the hope that we can shed some light on the problems of identifying and speciating human papillomaviruses in cervical lesions.

The problem, cervical carcinoma, is a serious one. It is clear from molecular and epidemiologic studies that HPV's play a role in the development of cervical cancer and that there is a subset of these viruses (the high risk group) that strongly correlates with the development of disease. A 1980 census suggests that there were 465,000 cases of cervical carcinoma in the world and that cervical cancer is the 2nd most frequent form of cancer in women representing about 15% of all malignancies. Best estimates from outmoded data banks suggest that cervical carcinoma is the #1 cause of female cancer in the developing world. The incidence of the disease in the US is 8cases/ 100,000 and that translates to about 13,500 new cases/yr. In contrast in Brazil, the country with the highest incidence in the world, the rate is 10 - fold higher reaching 83.2 cases/100,000. It should be clear to you that cervical cancer accounts for a significant amount of morbidity and mortality throughout the world.

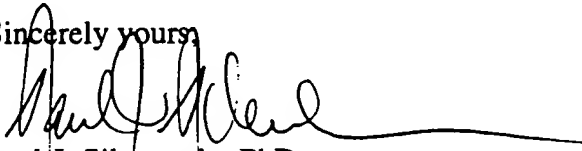
The problem is conceptually relatively simple, our task is to identify HPV sequences derived from one or another of the high risk group viruses (HPVs 16, 18, 31, 33, 45) in cervical lavages or biopsies. It is not necessary to speciate outside of the high risk group as the patient population with pathologically abnormal tissue will be treated regardless of virus type. Where typing becomes more important is in that population with no obvious lesion or with a low grade lesion where the virus turns out to be a high risk strain. We have, as you know, developed a rapid method for speciation that is based on amplification of the highly conserved L1 region of HPVs using the consensus primers developed by Manos and her colleagues. This first generation assay is excellent for incidence analysis and can be further developed for typing by restriction endonuclease cleavage of the amplified gene product. We have successfully applied the PCR - RFLP technique to the analysis of over 500 clinical samples that we have obtained from Dr. Tom Wright, my collaborator here at Columbia. We believe that the PCR - RFLP technique has gone as far as we can take it. The technique is wonderful at identification thus positive samples are truly positive, where we run into trouble is in lesions where more than a single virus type is present. Depending on the

-2-

abundance of one or the other genome there is a considerable degree of selective amplification even in the highly conserved L1 region that causes problems in homogenous amplification of all types within a sample. Therefore, we have spent the better part of the last year developing new technology based on amplification of the more divergent E6 region of the HPV genome. We focused on E6 because of its interaction with p53 and the clear implication in the carcinogenesis process. Our goal is to identify and speciate using a simple multi - well plate based hybridization assay and fluorescent readout. This approach is reasonably cost efficient and accurate. However, I am uncertain of its sensitivity at this time. With your input we would like to pursue the applicability of PCR/LDR technology to the problem at hand.

We are of course willing to share, primers, hybridization oligomers, sequence information and samples with you in an attempt to develop a sensitive, rapid approach to detection and characterization of HPV types within cervical tissue samples.

Sincerely yours,

A handwritten signature in black ink, appearing to read 'Saul J. Silverstein', with a long horizontal flourish extending to the right.

Saul J. Silverstein, PhD.  
Professor and Acting Chairman of  
Microbiology



National Institutes of Health  
National Cancer Institute  
Bethesda, Maryland 20892

January 18, 1994

Dr. Francis Barany  
Department of Microbiology  
Cornell University Medical College  
1300 York Avenue, Box 62  
New York, NY 10021

Dear Francis,

I was very pleased to hear from you, and I read your proposal "New Methods for Cancer Detection" with great interest and enthusiasm.

As you know, we have recently been applying the ligase chain reaction technique to study expressed homeobox genes in human breast cancer. We have identified several base changes in expressed HOX genes in a breast cancer-derived cell line. The pertinent questions now are (1) whether these base changes are polymorphisms or mutations, (2) whether they play a role in the biology of breast cancer, and (3) whether they may be useful diagnostically or prognostically. Your approaches to improve on the LCR technology would be extremely useful to us in our studies. We are now attempting to apply our LCR assay for a base change affecting the translational stop codon of HOXB7 to intact human breast cancer tissues, as well as to develop LCR assays for other polymorphisms/mutations that we have detected in HOXA4 and HOXC6. Your assistance and collaboration with us would be of enormous benefit.

I am enclosing my Biographical Sketch.

With best wishes for your success,

Sincerely yours,

*Mark*

Mark E. Sobel, M.D., Ph.D.  
Chief, Molecular Pathology Section  
Laboratory of Pathology  
National Cancer Institute  
Tel.# 301-496-7999  
Fax.# 301-402-4094  
E mail = molpath@helix.nih.gov

# Mayo Foundation

Rochester, Minnesota 55905 Telephone 507 284-2511

Mayo Clinic Mayo Medical School

Mayo Graduate School of Medicine

Steve S. Sommer, M.D., Ph.D.

*Department of Biochemistry  
and Molecular Biology*

December 8, 1993

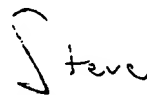
Dr. Francis Barany  
Associate Professor of Microbiology  
Department of Microbiology  
Cornell University Medical College  
1300 York Avenue, Box 62  
New York, NY 10021

Dear Francis:

Good luck on your DOD grant! I am enclosing our paper about dideoxy fingerprinting and also a review of phage promoter-based methods that will be coming out in early 1994. Since we speak IBM Wordperfect here, I enclose a hard copy of my NIH biosketch and I'll remind Jack Kovach to do the same. I also enclose a hard copy of an abstract page with relevant information.

Please note my correct fax number; the number that you have used previously will delay receipt of your faxes.

With kind regards,



Steve S. Sommer, M.D., Ph.D.  
Associate Professor of  
Molecular Biology  
Telephone (507)284-6033  
Telefax (507)284-3383

SSS:mj  
Enclosures



9/16/93

Dr. Francis Barany  
Department of Microbiology  
Cornell University Medical College  
1300 York Avenue  
New York, NY 10021

Dear Dr. Barany,

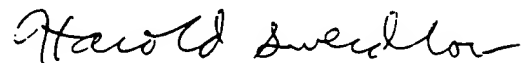
I am writing this letter to support your application for a program project grant from the National Cancer Institute. The informal collaboration we have recently begun has already been quite useful for me, and I am sure it will continue to be productive in the future.

As you know, my work has been focused on using capillary electrophoresis to improve the speed and efficiency of DNA-based diagnostics. We have constructed a prototype capillary PCR reaction and analysis instrument which can cycle, purify, load and analyze a DNA amplification reaction in only 30 minutes. Although we have not yet begun to multiplex and pipeline this instrument, it is already far faster than any other technology for diagnostics.

Recently, we have had some positive results performing LCR in a capillary format, and are encouraged by the data. Currently a 30 cycle LCR reaction takes over an hour to complete, however, we will be exploring the possibility of coupling PCR to a few cycles of LCR, in cooperation with your efforts. The potential of such a system for rapid, exquisitely accurate, early diagnosis of cancer is enormous.

The instrumentation we are working on should benefit the efforts you are currently undertaking, and I will help in any way possible to create an orchestrated diagnostic system that is simultaneously rapid, reliable and inexpensive.

Sincerely,



Dr. Harold Swerdlow  
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Paris, 16/01/94

Dr. F. Barany  
Cornell University Medical School  
Dpt of Microbiology  
1300 York Avenue, Box 62  
New York NY 10021  
USA

Dear Francis,

I am very pleased to confirm that I totally agree to begin a full collaboration with your group at the Cornell University Medical School.

As you know, I have been involved in the p53 field for now 8 years and our experience is rather important.

p53 alteration is the most common genetic alteration in human cancer. Briefly, 50 percent of human cancer harbor a p53 mutation. Furthermore, it is now clear that patients with such alteration have a worst prognosis, especially in breast carcinoma.

My group has been involved in the development of various approach to detect p53 alterations in tumor sample and their application in clinical use. Immunohistochemical and serological analysis have been set up and they have been transfer easily to clinical laboratories. Molecular analysis is still restricted to basic research unit as the sequencing of several thousand base pairs of a PCR product is still time consuming and cannot be use as routine so far. Molecular analysis of p53 alteration is necessary for some studies such as epidemiological analysis.

The LCR/LDR technology that you have developed can be a breakthrough in the molecular analysis of p53 alteration for the following reasons:

- 1) fast and easy detection of p53 mutations which can be automated and then use in clinical routine
- 2) use of very small amount of material which shows that such analysis can be performed either on archival material but also in small biopsies.
- 3) detection of p53 mutation in heterogeneous population.

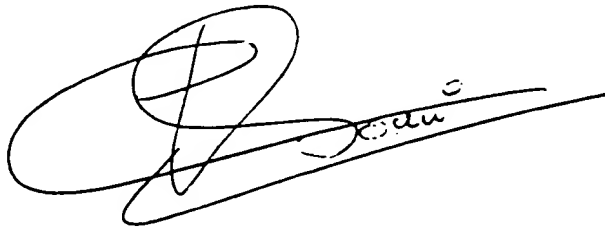
This third point is important for several reasons: i) detection of p53 in a small subset of cells in an early tumor indicates that such population would overgrow the other cells and lead to a more aggressive tumor. ii) in several cancer types, p53 mutation is a very early event which could be very important in the growth of the tumor (lung cancer especially). iii) it should be possible to find such alterations in tumoral circulating cells circumventing the need of biopsy.

As you know, I have managed a data base of p53 mutations which include more than 2500 mutations either published in the literature or as personal communications by several investigators. The statistical analysis of such data base should be very helpful for the development of your assay.

Finally, our laboratory is working with the hôpital St Louis where we have an easy access to biological material.

I really look forward for this collaborative work as it will be an important contribution to new diagnostic procedure of p53 alteration and in the near future to other genetic alteration.

Sincerely,

A handwritten signature in black ink, appearing to be 'T. Soussi', with a large, stylized circular flourish at the beginning.

Pr. Soussi T., Ph. D.  
Professor of Biochemistry  
University P.&M. Curie  
U301 INSERM

## THE NEW YORK HOSPITAL-CORNELL MEDICAL CENTER

DEPARTMENT OF PEDIATRICS  
DIVISION OF PEDIATRIC ENDOCRINOLOGY

September 8, 1993

Francis Barany, Ph.D.  
Department of Microbiology  
Cornell University Medical College  
1300 York Avenue  
New York, NY 10021

Dear Francis,

This letter is to confirm my continued enthusiastic collaboration with you on development of a PCR/LDR assay for diagnosis of congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency.

This disorder of cortisol biosynthesis occurs in a severe, "classic" form in approximately 1/10,000 births. About two-thirds of such patients are unable to synthesize aldosterone and, if untreated, they may die shortly after birth from excessive renal sodium loss and potassium retention. Accumulated cortisol precursors in the adrenal cortex are shunted into the pathway for androgen biosynthesis, so that patients have signs of androgen excess. Thus, affected females are born with ambiguous genitalia, a condition which often requires extensive surgery before normal sexual and reproductive function is achieved. Both males and female grow rapidly as children but have an advanced bone age, leading to short adult stature. This disorder is treated with replacement doses of hydrocortisone (cortisol) or another glucocorticoid and with a synthetic mineralocorticoid, fludrocortisone.

A milder, "nonclassic" form occurs extremely frequently (in up to 3% of certain ethnic groups such as Eastern European Jews). Affected individuals may be entirely asymptomatic or may develop signs of androgen excess during childhood or at puberty.

The ambiguous genitalia in affected females can be ameliorated by administering dexamethasone to the mother of each affected female fetus. This glucocorticoid crosses the placenta and suppresses secretion of androgens by the fetal adrenal gland. To avoid unnecessary treatment of males and unaffected females, accurate prenatal diagnosis is required.

Because affected males appear normal at birth, they are difficult to diagnose. To minimize the possibility of neonatal death, neonatal screening of Guthrie card blood spots has been advocated.



Although hormonal methods have been used for both prenatal and neonatal diagnosis, they are of limited accuracy and are not usable with prenatal treatment (which suppresses the hormonal abnormalities). Molecular diagnosis with linked polymorphic markers is also possible (the affected gene, *CYP21*, is in the *HLA* major histocompatibility complex) but is laborious and has a 1-2% error rate due to recombination.

My laboratory has demonstrated that 95% of mutant alleles can be detected by direct screening for the nine most common mutations, all of which involve recombinations (deletions and gene conversions) between the normally active gene, *CYP21*, and an adjacent pseudogene, *CYP21P*. We specifically amplified *CYP21* without amplifying *CYP21P* using the polymerase chain reaction and specific primers. We then identified mutations using allele specific oligonucleotide hybridization (ASO).

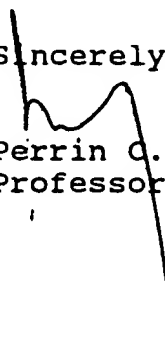
Although this approach is accurate, it is too laborious (a Southern blot plus 18 ASOs) for routine clinical use. I am thus very excited about applying the ligase detection reaction technique to this problem because of the possibility of multiplexing all of the necessary reactions. This will dramatically decrease the time and effort involved in genotyping and should allow nationwide implementation of prenatal diagnosis and eventually neonatal screening for this frequent genetic disorder.

As you know, the National Institutes of Health is enthusiastic about the progress that has been made in understanding this disorder and recently approved my grant on this subject with a priority score in the top 0.5%ile.

I anticipate that our collaborative studies will serve as a prototype for similar diagnostic approaches in other genetic diseases.

I wish you the best of luck with your grant application.

Sincerely,



Perrin C. White, M.D.  
Professor of Pediatrics



A Division of Perkin-Elmer Corporation

850 Lincoln Centre Drive, Foster City, California 94404 U.S.A. • Tel. (415) 570-6667 • Fax (415) 572-2743

September 29, 1993

Francis Barany, Ph.D.  
Department of Microbiology, Box 62  
Cornell University Medical College  
1300 York Avenue  
New York, NY 10021

Dear Francis:

I am writing this letter in support of your grant application entitled *New Methods for Cancer Detection* to the National Institutes of Health/National Cancer Institute. Applied Biosystems Division of Perkin Elmer Corporation (AB/PE) supports your efforts to develop a novel approach to detection of point mutations for genetic disease and cancer detection. A generalized method for mutation screening would have broad application in both these fields.

During the first two years of the five year collaboration between your laboratory and our company we have worked together on ligase-based mutation detection. We have supported work in your laboratory both financially, and by placing both a DNA synthesizer (Model 392) and a fluorescent DNA sequencer (Model 373A) at your disposal. In addition, we have already begun discussion of an upgrade of your DNA synthesis capacity. To date under this collaboration your group has focused on basic characterization of Taq ligase, and more recently on the feasibility of the PCR/LDR approach to assaying mutations in the 21-hydroxylase gene.

During this same timeframe AB/PE has worked out the concept of mobility modifying tails for high density multiplex mutation detection and developed a 30-mutation screening panel for cystic fibrosis mutations as our model system. This work has utilized the polymerase chain reaction, a technology which Perkin Elmer has a long history of commercializing, and the oligonucleotide ligation approach to mutation detection which was invented in 1984 at Applied Biosystems. Detection is done using our patented four-color fluorescent electrophoresis technology on our DNA sequencer. We have put our corporate expertise in DNA synthesis, sequencing, amplification, fluorescent detection, and data analysis into the development of this approach, and are pleased with the progress. This system will be unveiled at the American Society of Human Genetics Annual Meeting on October 8, 1993.

The next tools needed are methods to screen for unknown mutations such as those which occur in the p53 gene during carcinogenesis. This problem is further compounded by the need to be able to detect mutations which occur with low frequency in a given cell population. The approach you propose in your grant application has the potential to meet both of these needs, and we support your efforts to turn this idea into reality. We continue to have a strong interest in commercialization of a generic mutation screening system and hope that our collaboration on this project can result in a Perkin Elmer product.

Sincerely,

A handwritten signature in cursive script that reads 'Emily S. Winn-Deen'.

Emily S. Winn-Deen, Ph.D.  
Staff Scientist  
Human Genetic Analysis

**H. ACRONYM      DEFINITION**

Address	Position on an array made up of DNA or PNA which is complementary to a zip code primer.
Addressable array	A matrix of DNA or PNA oligomers which may be used to capture LDR or LCR products indicating the presence of a mutation by the position of fluorescent signal in the array.
APC	Adenomatous polyposis coli gene
BRCA1	Hereditary breast cancer gene
c-H-ras1	A cellular proto-oncogene with homology to a family of viral and cellular transforming oncogenes, (located on chromosome 11p).
Complementary zip code	DNA or PNA sequences perfectly complementary to zip code primers, which are used as "addresses" for detection of PCR/LDR, PCR/LDR/LCR, or PCR/RE/LDR products.
Convertide	Nucleotide analogue used to convert a wild-type sequence into one which contains a restriction endonuclease recognition site.
CYP21	Gene encoding 21 hydroxylase (located on chromosome 6p). Mutations in this gene are the most common cause for congenital adrenal hyperplasia.
CYP21P	Inactive pseudogene of 21 hydroxylase located near CYP21. Gene conversion between CYP21P and CYP21 are the major cause of 21 hydroxylase deficiency.
DCC	Deleted in colorectal carcinoma gene
DBMS	Database Management System
DGE	Denaturing gel electrophoresis
ERCC	Excision repair cross complementating gene(s)
FACC	Fanconi anemia complementation group C gene
Fam	6-carboxyfluorescein; fluorescent dye used in sequencing and mutation detection
FAP	Familial adenomatous polyposis syndrome
FMR-1	Fragile X mental retardation 1 gene
G6PD	Glucose 6-phosphate dehydrogenase (gene on chromosome Xq.)
GDB	Genome Database (Johns Hopkins University)
HER-2/neu	HER-2/neu/erbB oncogene (located on chromosome 17q.)
int-2	int-2 oncogene (on chromosome 11q.)
isoschizomer	Two restriction endonucleases which have the same recognition sequence.
K-ras	A cellular proto-oncogene with homology to a family of viral and cellular transforming oncogenes, (located on chromosome 12p).
LCR	Ligase Chain Reaction (uses four primers for exponential amplification of DNA with single base discrimination.)
LDR	Ligase Detection Reaction (uses two primers for linear and proportional amplification of DNA with single base discrimination.)
LS180	Colorectal adenocarcinoma cell line (carrying wild type p53 codon 248; mutant ki-ras codon 12)
MEN2a	Multiple endocrine neoplasia type 2a gene (also known as the <i>ret</i> proto-oncogene)
MCC	Mutated in colorectal carcinoma gene
MSH2	<i>Mutator S</i> human homolog 2 gene
NAD	Nicotinamide adenine dinucleotide, (Co-factor for <i>Tth</i> ligase).

NF1	Neurofibromatosis type 1 (von Recklinghausen's) gene
NF2	Neurofibromatosis type 2 (bilateral acoustic) gene
N-myc	A cellular gene with homology to a family of viral and cellular transforming oncogenes.
NMN	Nicotinamide mononucleotide
OMIM	Online Mendelian Inheritance in Man (Johns Hopkins University)
Oncogene	A dominantly acting gene involved in the unregulated growth of cancers
p53	Tumor suppressor gene (on chromosome 17p.)
pbC-N1	Plasmid containing the wild type Harvey- <i>ras</i> gene
PBL	Peripheral blood leukocytes
PCR	Polymerase Chain Reaction (uses two primers for exponential amplification of DNA region.)
PNA	Peptide Nucleotide Analogue
Primer dimers	Unwanted side-products formed by extension of primers on each other in a PCR reaction.
Proto-oncogene	A normal cellular gene which can be activated (converted to an oncogene) by mutations.
pT24-c3	Plasmid containing the mutant (codon 12) Harvey- <i>ras</i> gene from transitional
RB	Retinoblastoma gene
RE	Restriction endonuclease (cleavage.)
Sensitivity	The smallest amount of a mutation which an amplification method can identify in the presence of excess of wild type sequence. (Also; limit of detection).
SOD	Super oxide dismutase (gene on chromosome 21q.)
Specificity	The ability of an amplification method to discriminate between different DNA sequences.
SSCP	Single strand conformational polymorphism
SW837	Lung adenocarcinoma cell line (carrying mutant p53 codon 248)
<i>Taq</i> I	Thermophilic restriction endonuclease with T↓CGA recognition sequence.
<i>Tth</i> HB8I	Thermophilic restriction endonuclease isoschizomer of <i>Taq</i> I.
<i>Tth</i> ligase	Thermostable ligase from <i>Thermus thermophilus</i> used for LDR and LCR reactions.
T <sub>m</sub>	Oligonucleotide melting temperature. Also applies for melting of DNA/PNA duplexes. For short oligonucleotides T <sub>m</sub> is approximately equal to 4 x (G+C) + 2 x (A+T).
Touch prep	A method for obtaining a small homogeneous sample of cancers cells by touching a glass slide to a fresh or frozen tumor.
Tumor suppressor gene	A gene involved in development, regulation of cell growth, DNA repair, DNA binding, and/or other cellular functions. Loss of these functions is associated with tumor development.
VHL	von Hippel-Lindau syndrome gene
WT1	Wilms' tumor 1 gene
Zip codes	Primer sequences which have no homology to either the target sequence or other sequences on the genome. Also Used as "handles" on primers for detection of the "correct" product from PCR/LDR, PCR/LDR/LCR, or PCR/RE/LDR reactions.



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**Project 1.**

**Genetic Markers of Lung and Colon Cancer**

**Project Leader: Vincent Wilson**  
**The Childrens Hospital**  
**University of Colorado School of Medicine**

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. **DO NOT EXCEED THE SPACE PROVIDED.**

In the last decade mutations in many oncogenes and tumor suppressor genes have been described in cancers. This knowledge, however, has not significantly changed the care of cancer patients. Do cancer mutations predict the behavior of tumors? To correlate mutations with clinical outcomes we need robust methods to identify many possible mutations. Can the early spread of cancer be determined by finding the mutations of a cancer cell in the bone marrow? To detect micrometastases or early cancers we must be able to detect a few cancer cells out of many normal cells.

To achieve these capabilities we have devised two technologies: polymerase chain reaction/ligase chain reaction (PCR/LCR) to survey tumors for a wide number of mutations simultaneously; and polymerase chain reaction/restriction endonuclease digestion/ligase chain reaction (PCR/RE/LCR) for detecting a few cancer cells out of many normal cells. When they are fully developed PCR/LCR should be able to detect tens to hundreds of mutations at a sensitivity of one in  $10^2$  or  $10^3$ . PCR/RE/LCR selectively amplifies a mutation sequence by removing normal sequence. PCR/RE/LCR has already detected one mutation-bearing cell out of  $10^7$  normal cells.

To demonstrate the feasibility of these methods our specific aims are to: (i) Develop a PCR/LCR multi-gene, multi-mutation detection system to simultaneously identify mutations in three codons of the *k-ras* oncogene and nine codons of the p53 tumor suppressor gene. Approximately half of colon cancers have these *k-ras* mutations. About 15 percent of lung tumors and about 21 percent of colon cancers have one of these nine p53 mutations. Using PCR/LCR to identify these mutations we will investigate 40 colon and 50 lung tumors. (ii) Refine PCR/RE/LCR to detect the above p53 mutations at sensitivities of one in  $10^7$ . We will first use PCR/RE/LCR to determine the natural background mutation rate in non-cancerous tissues. Then, for patients whose tumors had detectable p53 mutations, we will use PCR/RE/LCR to investigate lymph nodes, blood and bone marrow specimens for micrometastases.

PERSONNEL ENGAGED ON PROJECT, INCLUDING CONSULTANTS/COLLABORATORS. Use continuation pages as needed to provide the required information in the format shown below on *all* individuals participating in the project.

Name	WILSON, Vincent	Degree(s)	Ph.D.	Social Security #	REDACTED
Position Title	Associate Professor / Director	D.O.B.	REDACTED	Role on Project	Co-investigator
Organization	The Children's Hospital & Univ. of Colorado School Of Medicine			Department	Pathology
Name	REZNIKOV, Leonid	Degree(s)	M.D./Ph.D.	Social Security #	REDACTED
Position Title	Research Fellow	D.O.B.	REDACTED	Role on Project	
Organization	The Children's Hospital & Univ. Colorado Health Sciences Center			Department	Pathology
Name		Degree(s)		Social Security #	
Position Title		D.O.B.		Role on Project	
Organization				Department	
Name		Degree(s)		Social Security #	
Position Title		D.O.B.		Role on Project	
Organization				Department	
Name		Degree(s)		Social Security #	
Position Title		D.O.B.		Role on Project	
Organization				Department	
Name		Degree(s)		Social Security #	
Position Title		D.O.B.		Role on Project	
Organization				Department	

Principal Investigator/Program Director (Last, first, middle):		F. BARANY, Ph.D.
DETAILED BUDGET FOR INITIAL BUDGET PERIOD	FROM	THROUGH
DIRECT COSTS ONLY	94/12/01	95/11/30

PHS 398 (Rev 9/91) (Form Page 4) Page 206 DD  
Number pages consecutively at the bottom throughout the application. Do not use suffixes such



**BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD  
DIRECT COSTS ONLY**

PROJECT 1

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED			
			2nd	3rd	4th	5th
<b>PERSONNEL:</b>						
<i>Salary &amp; fringe benefits</i>						
<i>Applicant organization only</i>		\$54,450	\$56,628	\$58,893	\$61,249	\$63,699
<b>CONSULTANT COSTS</b>		\$0	\$0	\$0	\$0	\$0
<b>EQUIPMENT</b>		\$2,595	\$2,000	\$2,000	\$2,000	\$2,000
<b>SUPPLIES</b>		\$10,300	\$10,712	\$11,140	\$11,586	\$12,049
<b>TRAVEL</b>		\$1,200	\$1,248	\$1,298	\$1,350	\$1,404
<b>PATIENT CARE COSTS</b>	<b>INPATIENT</b>	\$0	\$0	\$0	\$0	\$0
	<b>OUTPATIENT</b>	\$0	\$0	\$0	\$0	\$0
<b>ALTERATIONS AND RENOVATIONS</b>		\$0	\$0	\$0	\$0	\$0
<b>OTHER EXPENSES</b>		\$3,000	\$3,120	\$3,245	\$3,375	\$3,510
<b>SUBTOTAL DIRECT COSTS</b>		\$71,545	\$73,708	\$76,576	\$79,560	\$82,662
<b>CONSORTIUM/ CONTRACTUAL COSTS</b>		\$28,959	\$30,117	\$31,322	\$32,575	\$33,878
<b>TOTAL DIRECT COSTS</b>		\$100,504	\$103,825	\$107,898	\$112,135	\$116,540
<b>TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD</b>						<b>\$540,902</b>

(Item 8a)-&gt;

**JUSTIFICATION (Use continuation pages if necessary):**

**From Budget for Initial Period:** Describe the specific functions of the personnel, collaborators, and consultants and identify individuals with appointments that are less than full time for a specific period of the year, including VA appointments.

**For All Years:** Explain and justify purchase of major equipment, unusual supplies requests, patient care costs, alterations and renovations, tuition remission, and donor/volunteer costs.

**From Budget for Entire Period:** Identify with an asterisk (\*) on this page and justify any significant increase or decrease in any category over the initial budget period. Describe any change in effort of personnel.

**For Competing Continuation Applications:** Justify any significant increases or decreases in any category over the current level of support.

**Personnel:** Cornell University Medical College and Strang Cancer Prevention Center salaries are in accordance with the high cost of living in New York City, as well as the experience of the personnel. A 10% effort by the Principal Investigator and a 5% effort by Co-investigator assures full supervision of the junior personnel in this project. The Co-investigator will increase his effort and salary to 10% for years 2-5 reflecting increased responsibility in the project as our testing of breast biopsies expands. Cornell University Medical College has granted the Principal Investigator a Hirsch/Monique Weill-Caulier Career Scientist Award from 01/01/92 to 01/01/1997. This award of \$20,000 / year may be used as salary (and fringe benefit support) only. It thus allows the P.I. to spend full effort on research.

Dr. Jianying Luo is a research associate (Ph.D.) who has been in the Principal Investigator's laboratory since 9/01/92. Dr. Luo obtained her Ph.D. in the laboratory of Dr. Joseph Krakow at Hunter College. She is a highly skilled member of the Principal Investigator's laboratory. She has constructed,

Additional X-ray film cassettes with intensifying screens are requested to enable extended exposures of gels without having to wait for completion of previous studies. These equipment items will not only be dedicated for this project, but will also allow this work to progress more rapidly and efficiently.

**Supplies:** The costs listed under expendable supplies have been itemized and should realistically reflect our needs. Numerous oligonucleotides will be required for this project, as well as Taq polymerase. Although, extensive culturing of cell lines will be required to provide sufficient positive and negative controls for these studies, only minimal medium, serum, and tissue culture supplies are requested. Biochemicals, enzymes (proteinase K, RNase, restriction enzymes, etc.), X-ray film, agarose, polyacrylamide, and other electrophoresis supplies have been grouped together along with liquid Nitrogen (for snap freezing and storage of surgical tissue samples held for DNA isolation, and for cell lines), and radioisotopes ( $^{32}\text{P}$ ).

**Travel:** One trip per year is requested in order to present results of these studies and to network with other individuals on the latest advances in cancer.

**Other Expenses:** Support for publication and page charges, photocopying, library expenses, telephone, postage, and incidental office supplies is requested.

**B) Additional Years:** Funds requested for personnel have been adjusted by 4% per year to offset the estimated increases in salary and benefits. All other expenses have been similarly increased by 4% per year to offset inflation. Equipment costs are presently confined to the first year.

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**RESOURCES AND ENVIRONMENT**

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FACILITIES: Mark the facilities to be used at each performance site listed in Item 9, Face Page, and briefly indicate their capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Use "Other" to describe the facilities at any other performance sites listed in Item 9 on the Face Page and at sites for field studies. Use continuation pages if necessary. Include an explanation of any consortium/contractual arrangements with other organizations.

- Q Laboratory: The Molecular Genetics/Oncology lab comprises two separate rooms consisting of approximately 650 sq. ft. and is equipped with a fume hood (volatile carcinogens and isotopes), and a walk-in cold room and dark room (shared space).
- Q Clinical: Both the Veterans Administration Hospital and the University Hospital are located on the University of Colorado Health Sciences Center Campus. The Children's Hospital is a modern, accredited, well equipped facility affiliated with the University of Colorado, and has 218 beds, 16 of which compose the Oncology unit.
- Q Animal:
- Q Computer: Computers available: 1) Two Macintosh II computers, one of which is totally dedicated to clinical testing services (DNA diagnostics); 2) IBM AT which is dedicated to an extensively used HPLC
- Q Office: Vince Wilson's office (approximately 100 sq. ft.) is in good proximity to the laboratory in the Pathology Department at The Children's Hospital. Dennis Ahnen's office is located in the VA MC Hospital.
- Q Other (.):

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

Mol. Genetics/Oncology Lab: Thermal Cyclers: Perkin-Elmer Cetus 4800 (2) and Perkin-Elmer 9600, and Bioscler (cycler oven); Other equipment: Spectrophotometer, Speed-Vac/Lyophilizer, High Speed Centrifuge, Microfuges (3), HPLC, Liquid Scintillation Counter, Nucleic Acids Extractor (Model 341, ABI), assorted Power Supplies and Electrophoresis apparatus including Sequencing unit, assorted Temperature Baths and Heating Blocks, assorted refrigerators and freezers including a -80°C freezer, Liquid Nitrogen Freezer, and Tissue Culture Incubator.

Shared Facilities (The Children's Hospital Research Center and the Cytogenetics Laboratory): Ultracentrifuge, Radioisotope "hot" room, and Tissue Culture hoods and facilities.

ADDITIONAL INFORMATION: Provide any other information describing the environment for the project. Identify support services such as consultant, secretarial, machine shop, and electronics shop, and the extent to which they will be available to the project.

Research collaborations and colon cancer specimen exchange between Dr. Dennis Ahnen (VA Hospital, Denver) and Vince Wilson's laboratories have been consistently taking place for the past year. The connection for exchange of collaborations and lung cancer specimens has been established between Vince Wilson and the University of Colorado Cancer Center SPORE core units (as described by the Pilot project funded by the University of Colorado Cancer Center SPORE grant). Vince Wilson is a member and active participant of the University of Colorado Cancer Center, including the Division for Cancer Prevention and Control.

## A. SPECIFIC AIMS

The purpose of this project is to develop sensitive and specific methods for detecting oncogenic point mutations in lung and colon cancers. The sensitivity of the polymerase chain reaction (PCR) will be combined with the specificity of the ligase chain reaction (LCR) to identify specific mutations in these diseases. Two variations on the technique will be developed, which will allow us to explore mutations in the tumors themselves and in tissues containing rare cancer or precancerous cells. These methods will be applied to lung and colon tumors and to other clinical specimens to detect early cancerous changes or metastatic spread of the disease.

(i) **Development of a polymerase chain reaction/ligase chain reaction (PCR/LCR) method for identifying mutations in tumors.** This laboratory has used a combined PCR/LCR method to identify a K-ras mutation in colon polyps. As a first step to generalizing this technology, we will expand the number of simultaneous assays to identify mutations in codons 12, 13, and 61 of the K-ras gene and nine of the most common p53 gene mutations that occur in lung and colon cancers. Using cell lines and "touch prep" DNA samples we will develop the conditions that allow us to multiplex these reactions. Our initial aim is to be able to detect mutations at these 12 codons in lung and colon cancers. PCR/LCR will be compared to using LCR alone to see which technique achieves our target sensitivity of detecting one cancer mutation in  $10^2$  to  $10^3$  normal cells. PCR/LCR will also be compared to the PCR/LDR technique used in Project 2. The technique will be used to explore the frequency of mutations in lung and colon cancers. The mutations found will be correlated with the clinical parameters of the patients' conditions. Oligonucleotide or peptide nucleic acid addressable arrays (See Project 5) will let us expand the technology to screen for dozens or hundreds of mutations simultaneously.

(ii) **Development of a polymerase chain reaction/restriction endonuclease/ligase chain reaction (PCR/RE/LCR) to detect cancer mutations in one cell per  $10^6$  or  $10^7$  normal cells.** By PCR amplifying a DNA segment and digesting normal sequences, mutations in restriction endonuclease recognition sites can be selectively amplified. This laboratory has successfully identified 10 H-ras mutation-bearing plasmids from a background of  $10^9$  wild type copies, which is equal to a detection sensitivity of one in  $10^8$ . Likewise a detection sensitivity of  $10^7$  was achieved, when one cell with a mutation in the first base of codon 248 in the p53 gene was identified out of  $10^7$  normal cells. In both cases these cancer-associated mutations eliminated an *Msp* I restriction endonuclease site; by repeatedly digesting wild type sequence, the mutation was selectively amplified. Mutation and normal DNA were finally distinguished by using LCR. This level of sensitivity will be necessary for detecting micrometastases or identifying early, preneoplastic cells in sputum or stool samples. We will generalize this technique to detect mutations in any restriction endonuclease site. Along with Project 2, we also aim to expand this technology to detect any mutation by using one or two "conversion" primers to amplify DNA sequences at a specific location (see Project 3). Normal DNA will be converted to a restriction endonuclease sequence and cleaved with the enzyme. DNA with the mutation will resist restriction and be selectively amplified. If any of the lung or colon cancers examined contain a mutation at one of the five p53 mutations that eliminate a natural restriction endonuclease recognition sequence, other available clinical specimens (e.g. peripheral blood samples) will be investigated with PCR/RE/LCR for the presence of circulating cancer cells. Equally important will be our investigation of the normal background rates of mutation at codons in these genes. DNA from various normal tissues will be tested by PCR/RE/LCR for the presence of mutations. In these initial studies the rate of mutation will also be tested in sputum and bronchoalveolar lavage samples from smokers and colonoscopic biopsy specimens from patients at risk for colon cancer. These investigations may help us understand the role of environmental exposures and familial factors in carcinogenesis.

## B. BACKGROUND AND SIGNIFICANCE

Carcinogenesis is associated with mutations of proto-oncogenes and tumor suppressor genes in cancer cells. One of the most common mechanisms accounting for oncogene and tumor suppressor gene mutations is single base substitution. More than 85 percent of mutations in the retinoblastoma and p53 genes found in tumors are point mutations [1-5]. This is also true for somatic mutations in other tumor suppressor genes such as the adenomatous polyposis coli (APC) gene, the "deleted in colorectal cancer" (DCC) gene, the "mutated in

colorectal cancer" (MCC) gene, the Wilm's tumor (WT1) gene, the neurofibromatosis 1 and 2 (NF1 and NF2) genes, the von Hippel-Lindau (VHL) gene, the multiple endocrine neoplasia type 2 (MEN2a, RET) gene, and the human homolog of the *mutator S* (MSH2) gene [5-14]. Similarly, germline errors in DNA repair genes such as the excision repair cross complementing (ERCC) genes of xeroderma pigmentosum and the Fanconi anemia complementation group C (FACC) gene are generally base substitutions [15-21]. The ability to detect a large number of potential point mutations simultaneously in genetic investigations of tumors is crucially important to expanding our basic understanding of cancer and the clinical significance of genetic alterations in tumors.

The importance and frequency of base substitution mutations in some proto-oncogenes has been well characterized. Mutations in the known activation sites (e.g. codons 12, 13, & 61) of the *ras* genes are well documented in human cancer. Kirsten-*ras* (K-*ras*) mutations are frequently found in colorectal, gastric, lung, and other tumors, while Harvey-*ras* (H-*ras*) mutations are more often found in breast cancer. N-*ras* mutations are often found in leukemias and lymphomas [2, 22-25].

The oncogenic potential of mutations in many other cancer-related genes is not characterized as well, but is an area of intense investigation. The effect a point mutation has on the biological activity of a tumor may be related to the location (the site within the tumor suppressor gene) and the type of mutation (e.g. transition or transversion) [4, 5, 25, 26]. Indeed, p53 proteins with mutations in exons 5 and 6 are immunogenic and capable of binding heat shock protein (hsp) 70. Mutations in exons 7 and 8 of p53 result in a non-immunogenic protein that does not bind hsp 70 [27]. The oncogenic potential of mutations may be reflected in the frequency with which they occur, since mutations with greater oncogenic potential would be selected more often in tumor formation. However, only a few hotspots have been identified in the best characterized tumor suppressor genes. More than 100 mutations have been identified in the p53 gene, only about 5 sites have been considered hotspots [4, 5, 28, 29]. A G → T mutation in the third base of codon 249 in the p53 gene has been identified as a potentially important factor in the etiology of hepatocellular carcinoma [30, 31]. This specific mutation occurs only infrequently in other types of tumors, whereas a C → T mutation in the first site of codon 248 is commonly found in a variety of human tumors [2, 4, 32]. Do these mutations tell us something about the mutability of the DNA of these tissues at these sites or do they reflect biological selection for tumors of these tissues, when a mutation occurs at a particular codon?

A full description of genetic alterations in tumors is a potentially enormous task. More than half of human cancers contain p53 mutations [4, 5, 33], while mutations in the retinoblastoma, NF1, and WT1 genes appear to be confined to a significant but narrower field of tumor types [11, 13, 34, 35]. It is possible that some mutational hotspots have not been recognized in some cases, because small numbers of tumors have been studied. To date however, no hotspots have been identified in the retinoblastoma gene [1, 34, 36]. The p53 gene has been well studied, but technology has not allowed us to search large numbers of tumors rapidly for mutations in most cancer-related genes. Thus, a fuller understanding of cancer genes in human tumors has yet to be revealed.

Aside from the long and laborious process of direct sequencing, there is no robust method for identifying the precise base change at a point mutation. Techniques for surveying genes for mutations have been developed in the last decade, including mutation specific oligonucleotide probing, chemical or enzymatic (RNase A) cleavage of base pair mismatches, allele specific PCR, and a mismatch amplification mutation assay [37-40]. However, each of these assays is limited because of the labor involved and number of gene sites that can be studied at one time. They also lack the sensitivity to identify mutations in a minority of cells in a sample. The PCR technique reported by Dr. Peter Cerrutti and co-workers will detect mutations at sensitivities of one in a million, but requires the laborious task of cloning and sequencing [41, 42]. Presently, most laboratories use either single strand conformational polymorphism (SSCP) or denaturing gel electrophoresis to find exons or regions containing point mutations. This is followed by sequencing to identify specific mutations [38, 39]. Unfortunately, SSCP and DGE fail to detect up to thirty percent of base substitutions. Rapid, automated sequencing techniques are likely to be more available in the future, allowing researchers to identify new mutations in more cancer genes. However, sequencing should not be the method of choice for surveying for previously identified, potential mutations in tumors. Nor could sequencing detect point mutations in one cell in a million or better—the level of sensitivity that would be needed to detect minimal residual disease or early metastases. Thus, new, rapid, accurate, and sensitive techniques for

detecting mutations in tumors and a few tumor cells in tissue specimens are needed. The PCR/LCR and PCR/RE/LDR methods described in this proposal and in Project 2 will fulfill these needs.

We wish to state clearly, that the techniques we aim to develop are designed to survey tumors and tissues for mutations that have been discovered and described by other methods. We acknowledge the rapid advances being made in the detection of new oncogenic mutations [43]. An elegant method which amplifies and clones large and small differences between two genomes shows great promise for identifying genetic alterations associated with tumor development [44, 45]. The existence of small deletions, insertions, and single base differences between normal and mutant genes can be detected by SSCP and rSSCP [38, 46, 47], RNase or chemical cleavage of heteroduplexes [48-50], or even direct sequencing [5, 51, 52]. Clever variations on sequencing, such as dideoxy fingerprinting (ddF), have already been used to detect 84 out of 84 different mutations with a very low rate of false positive results [53]. Modifications of our methods might also be developed for identifying new mutations (See Project 4). Again, these methods are designed to find new mutations, and are unable to detect a mutation present in only a small fraction of the DNA. However, we expect that mutations discovered by the above techniques would be added to panels of mutations that can be detected by our PCR/LCR and PCR/RE/LCR technologies.

PCR-based schemes for detecting single base substitutions include allele-specific PCR, nested PCR, PASA or double ARMS amplification [51, 54-59]. However, diagnostic use of these techniques is limited. PCR requires careful optimization to detect a cancer mutation in the presence of normal DNA. Additionally, PCR is not sufficiently robust to accommodate the simultaneous detection of multiple targets (known as multiplexing). When several targets are located on a single gene, such as the p53 gene, multiple PCR primers interfere with each other during amplification. The LCR, however, can be combined in a synergistic way with PCR to overcome these problems.

The Program Director of this Program Project proposal developed the ligase chain reaction (LCR), a powerful method for detecting single base mutations. In this assay *Tth* ligase discriminates between normal and mutant DNA and amplifies the sequence [60, 61]. This enzyme links two oligonucleotides when they anneal adjacently on a complementary target sequence at 65°C. A mismatch at the junction of the two oligonucleotides prohibits ligation thus distinguishing between two DNA sequences. The ligated product can serve as a template for another set of oligonucleotides, complementary to the first pair. If these anneal adjacent to each other on the product of the first ligation, they too will be ligated and a ligase chain reaction will proceed exponentially with thermal cycling. (See LCR figure in Overview.) Using only one pair of adjacent primers gives a linear amplification called ligase detection reaction (LDR). LCR and LDR are compatible with PCR and allow the multiplex detection of single base mutations without primer interference.

The ligase chain reaction is ideal for multiplexing. Since there is no polymerization step, several primer sets can ligate along a gene without interference. The optimal multiplex detection scheme involves a primary PCR amplification, followed by either LCR (using four primers for exponential amplification) or LDR (using two primers for linear amplification). This approach has been successfully applied to multiplex detection of cystic fibrosis [62-64], hyperkalemic periodic paralysis [65], and 21 hydroxylase deficiency (D. Day, P. White, and F. Barany, unpublished). In the 21 hydroxylase study, individuals are determined to be heterozygous or homozygous for any of the ten common gene conversions that cause that disease. Likewise, PCR/LDR multiplexing has been used to distinguish between 30 different cystic fibrosis mutations, even when mutations were separated by only a single codon [64].

The elegant work of Dr. Bert Vogelstein and colleagues, which has been subsequently verified by Dr. Curt Harris at the NCI, has demonstrated that *K-ras* mutations can be detected in the stool of colon cancer patients [66]; and Dr. Curt Harris, personal communication). This approach could accurately identify mutations at the one percent level and lower, but is technically challenging and requires a biological phage plating step. Since Dr. Vogelstein's work has shown that *K-ras* mutations occur early in the progression of colorectal cancer, the ability to detect these mutations in mucosal cells shed into the feces may provide an early marker to the formation of adenomas and possibly polyps in individuals who have no previous history of disease. An alternative approach requires exceedingly careful optimization of both primers and conditions for allele specific PCR amplification of *K-ras* mutations [67]. This method was able to detect a few cells with *K-ras* mutations from the blood of pancreatic carcinoma patients. It was significantly less able to detect transition

mutations than transversions. Nevertheless, these two examples demonstrate the feasibility and perhaps clinical utility of detecting rare cancer cells in clinical specimens.

Our method for detecting rare mutation-bearing cells is based on removing normal sequence while selectively amplifying the cancer mutation. This laboratory has identified 10 *H-ras* mutation-bearing plasmids from a background of  $10^9$  wild type copies, for a detection sensitivity of one in  $10^8$ . A detection sensitivity of  $10^7$  was achieved, when one cell with a mutation in the first base of codon 248 in the p53 gene was identified out of  $10^7$  normal cells. In both cases these cancer-associated mutations eliminated an *Msp* I restriction endonuclease site; by amplifying and repeatedly digesting wild type sequence, the mutation was selected. Mutation and normal DNA in the reactions were ultimately distinguished by LCR (please see Preliminary Results). We will generalize this technique to detect mutations in any restriction endonuclease site. Along with Project 2, we also aim to expand this technology to detect any mutation by using one or two "conversion" primers to amplify DNA sequences at a specific location. Normal DNA will be converted to a restriction endonuclease sequence and cleaved with the enzyme. DNA with the mutation will resist restriction and be selectively amplified. LCR will be used to detect the mutant sequence at the end of the amplification and selection process.

It should be recognized that Project 1 and Project 2 are working jointly to develop the two gene amplification and mutation detection methods that are the heart of this Program Project. In the interest of putting these technologies on the broadest possible foundation, though, the two projects will explore slightly different variations on the same methods, each of which may have its particular advantages. Project 1, working mostly with radioactive labeling, will use PCR/LCR and PCR/RE/LCR. Project 2, using fluorescent techniques primarily, will focus on PCR/LDR and PCR/RE/LDR. In both cases the PCR provides sensitivity (amplifying the mutation), while the LCR or LDR contributes specificity (discriminating between mutation and wild type). LCR has the attractive benefit of providing additional sensitivity to mutation detection, especially when signal strength may be weak. For example, detecting one cancer mutation-bearing cell in  $10^4$  normal cells might not require restriction enzyme selection (i.e. PCR/RE/LCR), if PCR/LCR can amplify the mutation signal sufficiently. LDR, on the other hand, requires two less primers and is more likely to aid in quantification, because it amplifies linearly. Project 2 will also explore the use of LCR alone to perform multiplex detection of mutations.

As described above in the Specific Aims, our goal is to develop two new, mutation-detecting technologies; one with a capacity to recognize tens (and potentially hundreds) of mutations at a low sensitivity (one in  $10^2$  to one in  $10^3$ ) and one with a high sensitivity (one in  $10^6$  to one in  $10^7$ ) but lower capacity. These techniques have the potential to make significant contributions to cancer research and clinical oncology. On a limited scale both methods might be put into practice right now. For a few families in which a cancer syndrome (e.g. Li-Fraumeni Syndrome) is inherited, gene-carrier status could be determined. Tumors could be investigated for the presence of specific mutations. This laboratory has identified a K-ras mutation in colon polyps using PCR/LCR technology. (See Preliminary Results.) Furthermore, our preliminary results also suggest that early metastases or minimal residual disease could be shown now; we have detected a mutation in codon 248 of the p53 gene in one cell out of  $10^7$  normal cells. The ability to detect and identify single point mutations at sensitivities of one cell in a million or more would offer clinical oncologists new tools to improve patient care.

The PCR/LCR technology that we propose here will be used primarily to examine tumor and constitutional DNA. To be able to explore the relationship between growth-controlling genes and the behavior of tumors we must be able to detect a very large number of potential mutations in many genes with ease, and be able to make correlations between the mutations and clinical outcomes. The ability to survey tumors for multiple mutations in multiple genes will make this possible. If the technology can be extended for use on fixed tissue, older pathology specimens could be studied and correlated with the current status of patients and the therapies they received. Our goal of a sensitivity of one in  $10^2$  to one in  $10^3$  will make it possible to recognize subclones of cells in tumors by their mutant gene signatures, allowing us to correlate these genetic alterations with distant metastases or resistance to treatment. Genetically determined host characteristics may also be important to identify before choosing a therapeutic option. For example, DNA testing has been used to determine histocompatibility [68, 69].



Oncological care in the future may be guided by the mutated genes identified in tumors and even by the site and type of mutation. Rapid and accurate mutation detection may become more important as advanced technologies such as gene therapy evolve [70-73]. PCR/LCR technology could also help distinguish a new primary tumor from the recurrence of a previous tumor, if the genetic signatures of the two cancers were known. In fact, genetic characteristics may help determine the origin of a tumor, when the histology is unclear [74]. As genes that predispose to cancer are discovered, screening for inherited cancer risks will become more feasible. We have not overlooked the fact that this technology and the PCR/RE/LCR technique are entirely applicable to detecting and identifying specific infectious disease agents. Rapidly identifying infectious agents and determining their antibiotic sensitivities could be lifesaving for many patients being treated for cancer.

The PCR/RE/LCR technology that we propose here will be used to identify cancer mutations in a few cells on a background of normal cells. This may have immediate application to clinical situations when the genetic changes in a primary tumor have already been determined. Minimal residual disease, the existence of micrometastases to lymph nodes or bone marrow and microinvasive disease could all be assessed. As the genetic changes of the early stages of carcinogenesis are discovered, screening for the premalignant phases of some cancers (e.g. the adenomatous polyps that precede colon cancer) may become feasible using this technology. Indeed, K-ras mutations have been found in DNA from the stool of patients with colon polyps, although the method that showed this relied on cloning and sequencing after PCR was applied [66]. For research purposes PCR/RE/LCR technology will allow us to explore the early stages of tumorigenesis, genetic changes associated with aging and may help us determine the effects of exposures to chemicals and other potentially harmful agents.

In summary, the gene-amplification, sequence-detection methods that we aim to develop will have broad applications in cancer research, clinical oncology and medicine in general. Specifically, PCR/LCR with its capacity to screen for many DNA sequences will be used to:

- (i) Detect germline mutations in hereditary cancer syndromes (e.g. Hereditary Non-polyposis Colorectal Cancer, Li-Fraumeni syndrome, Familial Polyposis, etc.).
- (ii) Determine host characteristics (e.g. histocompatibility) prior to therapy.
- (iii) Research the relationship between genetic alterations in tumors and clinical outcomes. Clinical decision making may eventually be guided by cancers' genetic characteristics.
- (iv) Distinguish second primary tumors from the recurrence of an initial tumor by determining their different genetic "signatures".

PCR/RE/LCR with its high sensitivity will be used to:

- (i) Screen for the presence of early or premalignant disease by identifying the early genetic changes of carcinogenesis in clinical specimens. Stool, sputum and bronchiolar lavage specimens, urinary specimens, and small biopsies (e.g. fine needle biopsies of the breast or other organ) could be assayed for point mutations to determine presence of a tumor or potentially tumorigenic cells.
- (ii) Detect microinvasive disease. A clonal mutation would insure the identification of invasion in neighboring surgically resected tissues when the margin of the tumor is difficult to decipher.
- (iii) Detect residual or early recurrence of disease to permit earlier therapeutic intervention. The monitoring of leukemia patients who carry a Bcr-abl gene rearrangement has been well documented [75, 76].
- (iv) Detect micrometastases in bone marrow or lymph nodes in breast, lung or colon cancer, especially where the micrometastases may be too small to be detected by conventional histopathology.
- (v) Research the early steps in carcinogenesis and the effects of potential carcinogens. The analyses of individuals for oncogenic changes as well as silent (apparently non-oncogenic mutations) may provide distinctive mutational spectra in exposed people [77].
- (vi) Detect somatic mutations associated with aging.



**C. PRELIMINARY RESULTS****(i) PCR/LCR detection:**

The sensitivity and specificity of the combined PCR/LCR technique is valuable for detecting base substitution mutations in cancers. Our goal is to develop a polymerase chain reaction/ligase chain reaction (PCR/LCR) method for identifying mutations in tumors. Our initial aim is to be able to expand this technology to identify mutations in codons 12, 13, and 61 of the K-ras gene and nine of the most common p53 gene mutations that occur in lung and colon cancers. PCR/LCR will be compared to using LCR alone to see which technique achieves our target sensitivity of detecting one cancer mutation in  $10^2$  to  $10^3$  normal cells. The technique will be used to explore the frequency of mutations in lung and colon cancers.

(a) *Detection of base substitution mutation in colonic polyp tissue.* We analyzed DNA isolated from seven human adenomatous colon polyps for mutations in codon 12 of the K-ras gene (See Fig. 1). Codons 12, 13 and 61 of the K-ras gene are frequently mutated in colon polyps and cancers [2, 25]. LCR primers were synthesized to recognize wild type and G → A or T mutations at codon 12 of K-ras. (A diagram of similar PCR and LCR primers, used for the detection of H-ras gene is shown in Fig. 3.) The cell lines LS180 and SKCO1 were used as controls for the G → A and G → T mutations respectively. Specimen #5744 was shown to contain a G → A mutation, while the other six specimens were negative. This is consistent with the frequency of K-ras mutations in colon polyps [2, 25, 26]. Since the positive signal is weak for sample #5744, it is possible that the mutant cells represent only a subpopulation of the polyp.

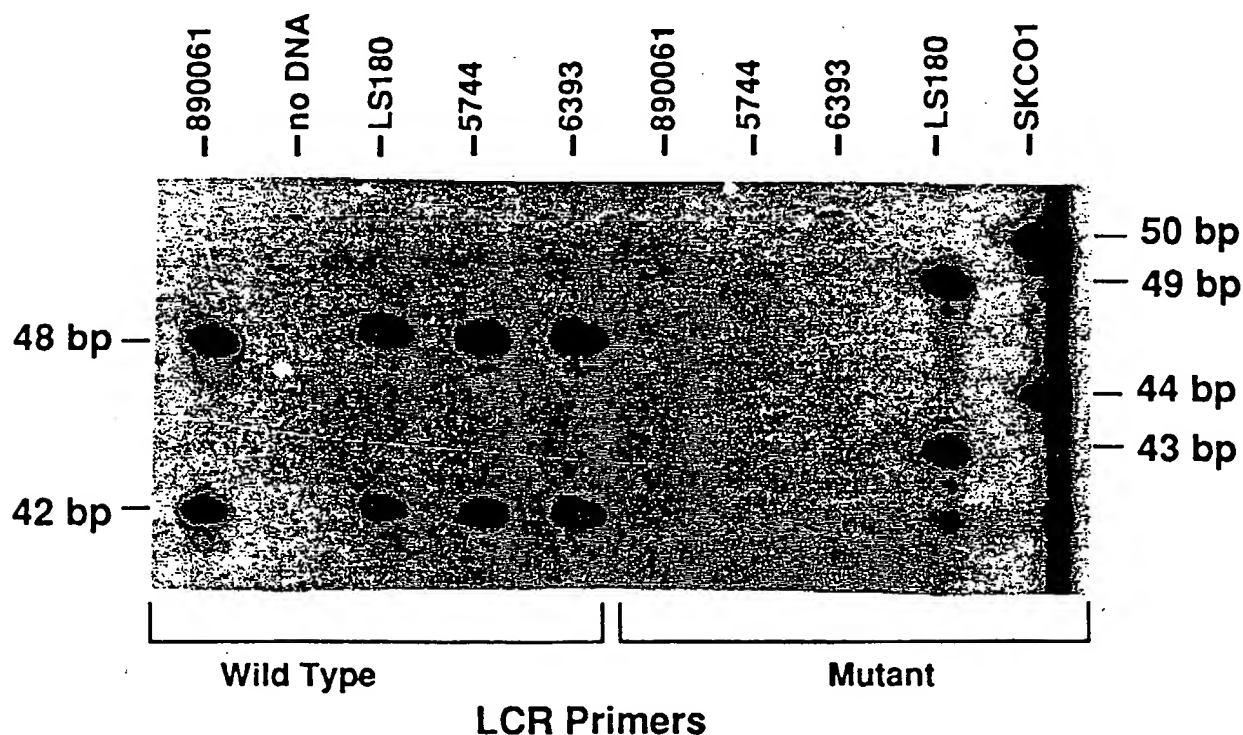


Fig. 1. Detection of a K-ras codon 12 mutation in colon polyps. Autoradiograph of human K-ras codon 12 LCR analysis of colon polyps and control DNAs, separated on a 10% polyacrylamide sequencing gel. The LCR primers were end-labeled with  $^{32}\text{P}$  prior to amplification. One  $\mu\text{g}$  of DNA was subjected to PCR amplification prior to LCR. The LCR primers were chosen to provide 42 bp and 48 bp fragment sizes for the sense and antisense strands of wild type DNA, 43 bp and 49 bp fragments for the codon 12 G → A mutation (GGT → GAT, LS180 cell line), and 44 bp and 50 bp for the codon 12 G → T mutation (GGT → GTT, SKCO1 cell line).

**(ii) PCR/RE/LCR detection:**

The enormous sensitivity and specificity of the PCR/RE/LCR technique is valuable for detecting base substitution mutations in a few cancer cells analyzed against a background of normal cells. Our goal is to develop a polymerase chain reaction/restriction endonuclease/ligase chain reaction (PCR/RE/LCR) to detect cancer mutations in one cell per  $10^6$  or  $10^7$  normal cells. By PCR amplifying a DNA segment and digesting normal sequences, mutations in restriction endonuclease recognition sites can be selectively amplified.

(a) *Plasmid DNA controls and in vitro detection sensitivity.* We began a series of experiments using the PCR/RE/LCR technique to fulfill the clinical and research need for a technique with the ability to detect point mutations at sensitivities of one in  $10^6$  or better. Codon 12 of the H-ras gene was chosen to begin this approach for two reasons: 1) Wild type control (pbc-N1, GGC) and mutant (pT24-c3, GTC) plasmids were easily available for testing and standardizing these procedures; and 2) The H-ras gene contains a *MspI* restriction site (CCGG) at the codon 12 locus (CCGGC), such that a mutation in either of the first two bases of codon 12 can be selected due to the loss of the *MspI* recognition sequence. Three sets of consecutively nested PCR primers were chosen for three cycles of amplification with *MspI* selection of the mutant alleles [41, 42]. Known mixtures of mutant and wild type plasmid were initially digested with *MspI* prior to PCR amplification with the outer most primers (Fig. 2, below). The amplified product was then subjected to *MspI* digestion again before a nested PCR amplification. Restriction and PCR amplification was repeated a third time. This was followed by a fourth *MspI* digestion. Such repeated amplification/restriction endonuclease digestion should selectively amplify mutant DNA, as well as unwanted potential products due to *Taq* polymerase errors during amplification (see below). The correct mutation may be properly identified by LCR amplification of the mutant allele (See Fig. 2.). LCR primers are designed to differ in length, such that mutant and wild type signal are distinguished when products are separated on a sequencing gel (see Fig. 3).

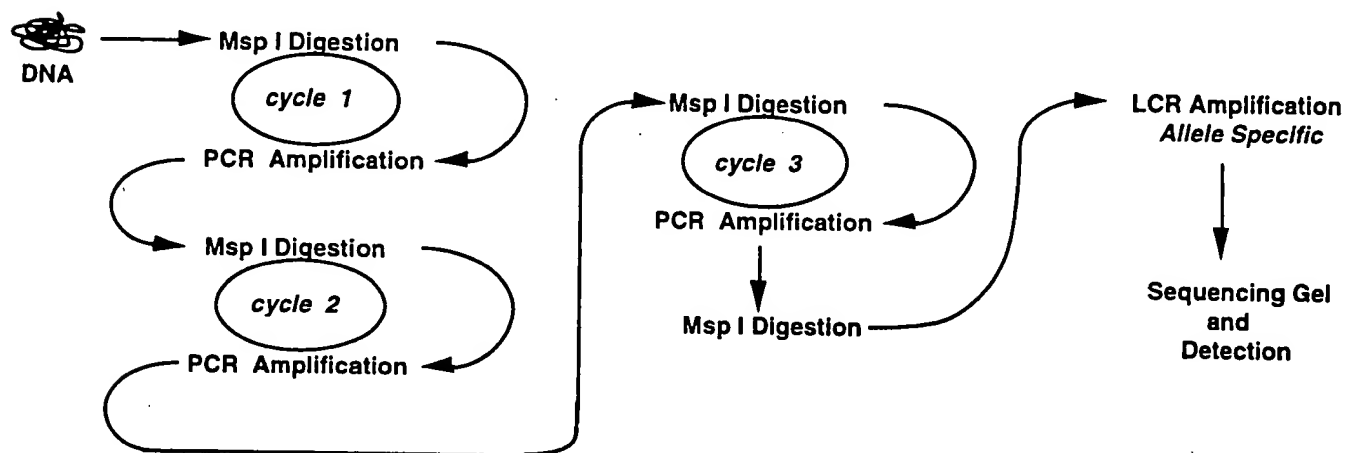


Fig. 2. Diagram of the PCR/*MspI*/LCR selection of mutations in H-ras codon 12 and p53 codon 248. DNA samples are subjected to three cycles of *MspI* restriction and PCR amplification followed by a final *MspI* restriction prior to LCR. The combined total PCR cycles was 65, followed by 30 cycles of LCR amplification. Discriminating primers specific for the wild type and the mutant (G → T) were picked to optimize the specificity of the LCR amplification process according to previously reported studies [60, 61]. The LCR amplification reaction was performed in the presence of radioactive ( $^{32}\text{P}$ ) end-labeled common primers, as previously described [60, 61]. LCR products were then separated on a 10% polyacrylamide sequencing gel and detected by exposure of the dried gel to X-ray film.

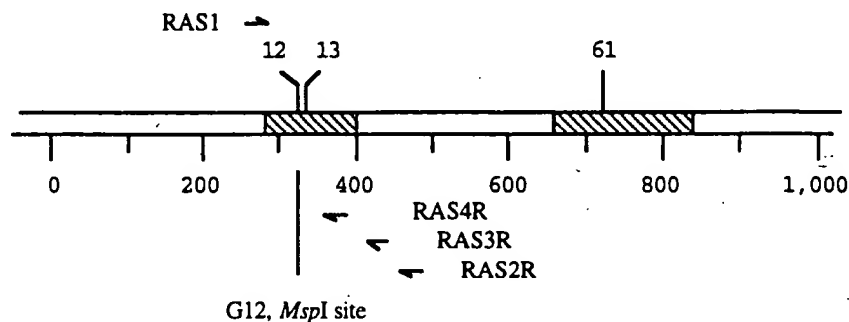
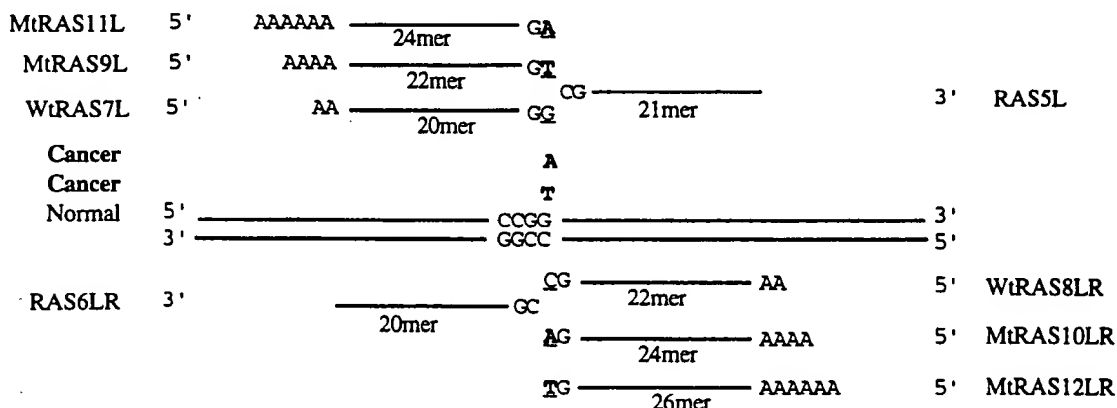
**1. PCR and biochemical selection reactions:****2. LCR reaction:**

Fig. 3. Diagram of the PCR and LCR primers used for selection of mutations in Ha-*ras* codon 12. 1. Position of primers RAS1, RAS2R, RAS3R, and RAS4R. Use of primers RAS1 and RAS2R specifically amplifies a region of the Ha-*ras* gene from human genomic DNA. After *MspI* selection, a smaller region is amplified using RAS1 and RAS3R. By nesting the PCR reaction, one avoids amplifying incorrect amplicons from the first round of PCR. 2. LCR detection of mutant sequences at codon 12. Primers WtRAS7L, RAS5L, RAS6LR, and WtRAS8LR are complementary to the wild type *ras* gene. These primers will amplify using LCR in the presence of wild type *ras* gene sequence. Primers MtRAS9L, and MTRAS10LR are complementary to the G12V mutation (G → T), while primers MtRAS9L, and MTRAS10LR are complementary to the G12D mutation (G → A). These primers will ligate to the common RAS5L and RAS6LR primers only in the presence of target DNA containing the appropriate mutation. Results are shown in Fig. 4.

The selection process described above allowed us to detect 10 copies of the mutant plasmid (pT24-c3) DNA in the presence of  $10^9$  copies of wild type (pbC-N1) plasmid. This is equivalent to a detection sensitivity of one mutant allele in  $10^8$  (See Fig. 4.). However, the sensitivity of detection was dependent on the number of cycles of *MspI* restriction and PCR amplification run prior to LCR analysis. Three cycles of *MspI* and PCR selection were required to achieve the one in  $10^8$  sensitivity. If only two cycles of *MspI* restriction and PCR selection were performed, the assay did not detect less than one in  $10^6$  (see Fig. 4). Thus, these procedures may be used semi-quantitatively by bracketing the detection of mutations with the number of selection cycles used. For a more quantitative approach, see use of "markers" in Project 2.

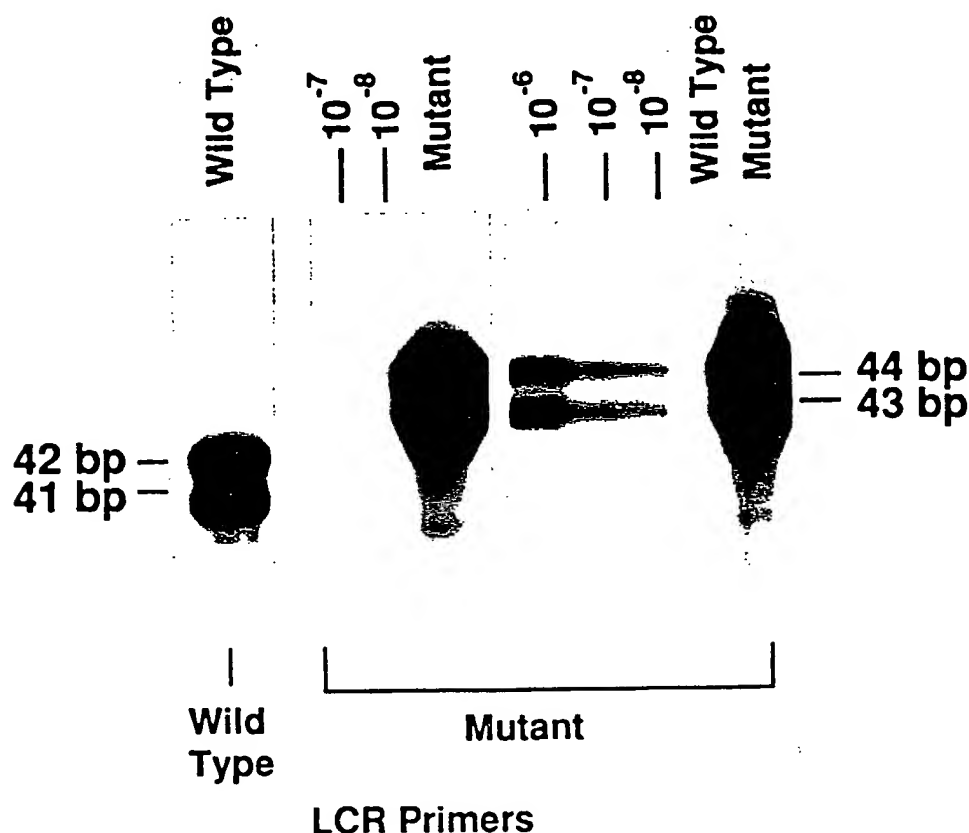


Fig. 4. Detection of human Ha-*ras* mutation using PCR/*MspI*/LCR. Autoradiograph of human Ha-*ras* codon 12 LCR analysis of control mixtures of mutant and wild type plasmid DNAs, separated on a 7 M urea, 10% polyacrylamide sequencing gel. The LCR primers were end-labeled with  $^{32}\text{P}$  prior to amplification. Control plasmids pT24-C3 (GTC, mutant) and pbCN1 (GGC, wild type) DNAs were mixed at the levels denoted and the mutant allele was selected by two and three cycles of *MspI* restriction digestion and PCR prior to LCR. The LCR primers were chosen to provide 41 bp and 42 bp fragment sizes for the sense and the antisense wild type DNA strands, and 43 bp and 44 bp fragments for the codon 12 mutation (GGC  $\rightarrow$  GTC).

(b) *High sensitivity detection in cultured cell lines.* Primers have also been chosen for a mutation at the first base of codon 248 (CGG  $\rightarrow$  TGG) in the p53 gene, which also encodes an *MspI* recognition site. The cell line LS180 has the wild type sequence at p53 codon 248, CGG. The cell line SW837 has the mutated sequence at p53 codon 248, TGG. (Reported by Nigro et al. [33], and verified by this laboratory.) Using the same PCR/*MspI*/LCR procedures described above for codon 12 of H-*ras*, a single mutant SW837 cell (p53 codon 248, TGG) in the presence of  $10^5$ ,  $10^6$  or  $10^7$  wild type cells (LS180) was detected. (See Fig. 5 for primers and Fig. 6 for results) Thus, the practical sensitivity of this technique is at least one mutant cell in  $10^7$ , depending on the DNA specimens available (e.g. 60  $\mu\text{g}$  is equivalent to  $10^7$  cells). These techniques work well in cell pellets and tissue specimens (as noted above in the analysis of colon polyps).

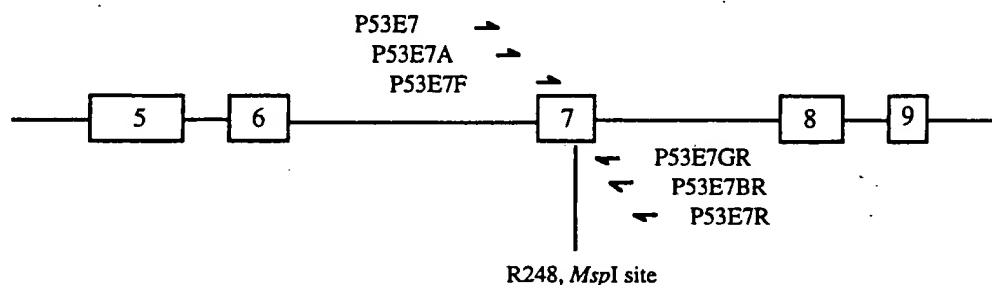
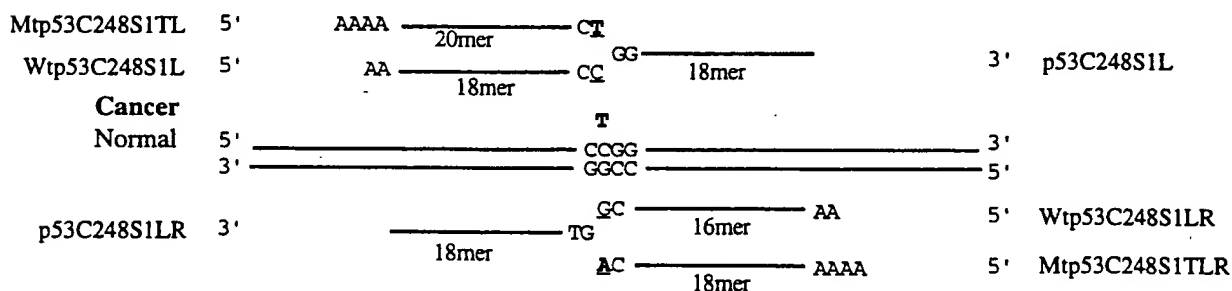
**1. PCR and biochemical selection reactions:****2. LCR reaction:**

Fig. 5. Diagram of the PCR and LCR primers used for selection of mutations in p53 tumor suppressor gene codon 248. 1. Position of primers. Primers P53E7 and P53E7R specifically amplify a region of the p53 gene from human genomic DNA. After *MspI* selection, a smaller region is amplified using P53E7A and P53E7BR. By nesting the PCR reaction, one avoids amplifying incorrect amplicons from the first round of PCR. Likewise, primers P53E7F and P53E7GR amplify an even smaller fragment of 240 bases which flanks codon 248. 2. LCR detection of mutant sequences at codon 248. Primers Wtp53C248S1L, and Wtp53C248S1LR are LCR primers whose 3' base is complementary to the wild type p53 codon 248. These primers will amplify using LCR in the presence of adjacent common primers p53C2481L, p53C248S1LR, and wild type target DNA. Primers Mtp53C248S1TL, and Mtp53C248S1TLR are complementary to the R248W mutation (C → T). These primers will ligate to the common primers p53C2481L, p53C248S1LR only in the presence of target DNA containing the R248W mutation. Results are shown in Fig. 6.

(c) *The question of Taq polymerase fidelity.* The misincorporation rate reported for *Taq* polymerase suggested that these procedures would never reach a detection limit of better than one in  $10^6$  [78, 79]. However, the fidelity of *Taq* polymerase was determined as the misincorporation at *any* base within a large amplified region. The fidelity is actually greater for any specific base. Our PCR/*MspI*/LCR procedures have failed to detect a mutation in more than 12 separate analyses of the H-ras wild type plasmid (pbC-N1) DNA. The risk of false positives due to polymerase misincorporation or mis-ligation cannot be ruled out and will be avoided by always running both positive and negative controls, as well as running clinical samples in duplicate and triplicate.

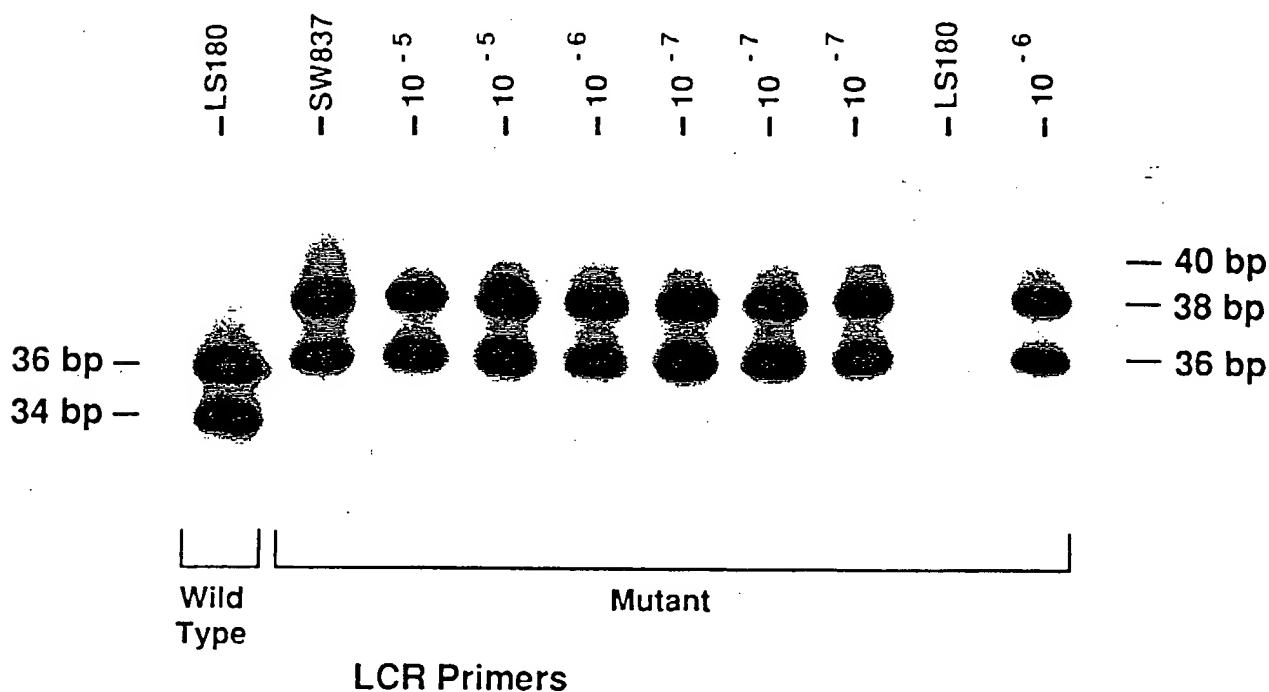


Fig. 6. High sensitivity detection of human p53 codon 248 mutation present at 1 in  $10^6$  or  $10^7$  normal cells using PCR/MspI/LCR. Autoradiograph of human p53 codon 248 LCR analysis of DNAs isolated from mixtures of mutant and wild type cell lines, separated on a 7 M urea, 10% polyacrylamide sequencing gel. The LCR primers were end labeled with  $^{32}\text{P}$  prior to amplification. The mutant cell line, SW837, contains a C  $\rightarrow$  T mutation in codon 248 (CGG  $\rightarrow$  TGG), while LS180 cells carry a wild type sequence in this genomic locus. Either ten SW837 cells were pelleted with  $10^6$  LS180 cells, or only a single SW837 cell was pelleted with  $10^6$  and  $10^7$  LS180 cells (as noted in the figure). The DNA was isolated from the cell mixtures and subjected to three cycles of MspI restriction digestion and PCR prior to LCR. LCR primers were chosen to provide 34 bp and 36 bp fragment sizes for the sense and antisense strands of wild type DNA, 36 bp and 38 bp for the codon 248 C  $\rightarrow$  T mutation (CGG  $\rightarrow$  TGG), and 38 bp and 40 bp for the codon 248 C  $\rightarrow$  A mutation (CGG  $\rightarrow$  AGG).

(d) *Human background mutation frequency.* Before positive results from these procedures can be interpreted as detecting the presence of a cancer mutation, a basic question must be answered: What is the background rate of single base substitution mutations in oncogenic loci in human tissues? We have begun to answer this question by analyzing peripheral blood leukocyte DNA from seven subjects for the G  $\rightarrow$  T transversion at the second base of codon 12 in the H-ras gene. As demonstrated in Table 1, six of the seven DNAs analyzed carried a mutation frequency of less than the detection limit. (Using 6  $\mu\text{g}$  DNA as starting material, representing approximately  $10^6$  cells, the detection limit of this assay can be, at best, one in  $10^6$ .) The seventh individual provided a positive result. Since, this person's G  $\rightarrow$  T mutation could not be detected after two cycles of PCR amplification and MspI restriction selection, the frequency of cells bearing this mutation is probably between one in  $10^5$  and one in  $10^6$ . Individual number 7 is being re analyzed with another aliquot of

6 µg of DNA to verify the presence of this mutation in his leukocytes. A better approximation of the frequency of the G → T mutation at the second base of codon 12 in the H-ras gene in human DNA will probably require PCR/*Msp*I/LCR analysis of 60 µg DNA, providing a sensitivity of one cell in 10<sup>7</sup>, and analysis of a larger number of blood specimens.

**TABLE 1**  
**ANALYSIS OF HUMAN PERIPHERAL BLOOD LEUKOCYTE DNAS**  
**FOR G → T MUTATIONS IN THE MIDDLE BASE OF H-ras CODON 12<sup>a</sup>**

Individual	Codon 12 Sequence	
	2 Cycles of Selection	3 Cycles of Selection
1	not done	wild type
2	not done	wild type
3	not done	wild type
4	not done	wild type
5	not done	wild type
6	not done	wild type
7	wild type	GTC

<sup>a</sup> 6 µg of DNA was subjected to either two or three cycles of restriction and PCR amplification prior to LCR analysis.

As noted above, these procedures have also been developed for the human p53 codon 248 (CGG). LCR primers were chosen for identification of the C → T, or A in the first position of codon 248. This first base in codon 248 is a 5-methyldeoxycytidine site and the high frequency of occurrence of C → T mutations in human genetic diseases has been theorized to be due to deamination of 5-methyldeoxycytidine [4, 25, 32, 71, 80, 81]. Thus, determining the background C → T mutation frequency of codon 248 of p53 in various tissues may be important to a fuller understanding of human oncogenesis.

### (iii) Publications:

These preliminary studies have been in part presented at one international and two national meetings and published as abstracts:

- 1) Wei, Q., Barany, F., & Wilson, V.L.: Sensitive detection of point mutations by combined PCR and LCR Techniques. Proceedings of the International Conference on Molecular Biology of Genetic Diseases, Shanghai, P.R. China, pp. 40, (1992).
- 2) Wei, Q., Barany, F., & Wilson, V.L.: Oncogenic point mutations detected by combined PCR and LCR Techniques. Mol. Biol. Cell 3 (supplement): 22a (1992).
- 3) Wilson, V.L., Wei, Q., Parker, N., Manchester, D.K., & Barany, F: Frequency of oncogenic mutations in human tissues determined by combined PCR and LCR techniques. Proc. Am. Assoc. Cancer Res. 34: 262 (1993).

## D. Experimental Design and Methods

Carcinogenesis is associated with mutations of proto-oncogenes and tumor suppressor genes in cancer cells. One of the most common mechanisms accounting for oncogene and tumor suppressor gene mutations is single base substitution. The ability to detect a large number of potential point mutations simultaneously in genetic investigations of tumors is crucially important to expanding our basic understanding of cancer and the clinical significance of genetic alterations in tumors. Ultimately, our purpose is to simultaneously characterize as many of these mutations as possible, to correlate tumor mutations with disease outcomes. To achieve our purpose we aim to develop two technologies:

(i) *a multiplex polymerase chain reaction/ligase chain reaction (PCR/LCR) system for detecting point mutations in tumor biopsies.* Expected result: Rapid and simultaneous detection of mutations in the three K-ras codons (found in approximately 50 percent of colon cancers [2]) and mutations in nine codons in the p53 tumor suppressor gene (found in approximately 15 percent of lung tumors and 21 percent of colon cancers [28, 29]).

(ii) *a multiplex polymerase chain reaction/restriction endonuclease/ligase chain reaction (PCR/RE/LCR) system for detecting point mutations at a sensitivity of one mutation in  $10^6$  or  $10^7$  cells.* Expected result: Detection of micrometastases, detection of precancerous cells in sputum and colorectal washings or stool.

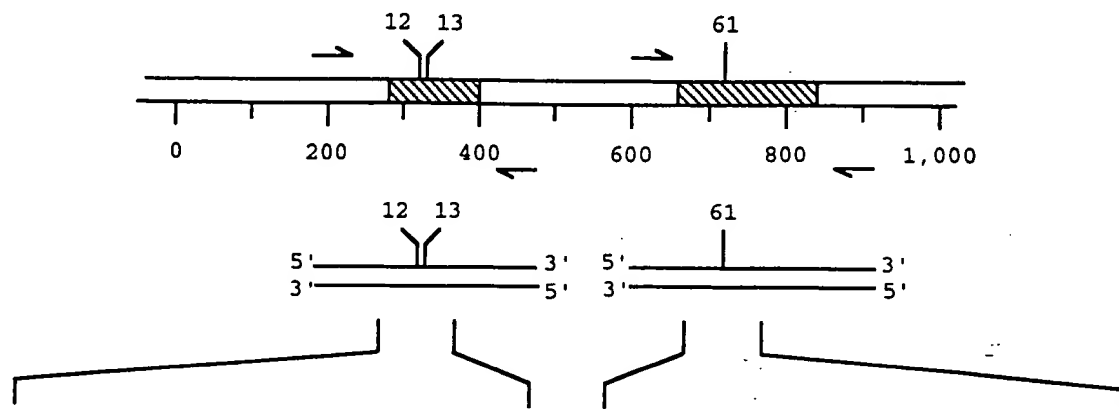
The Project Leader considers himself fortunate to be able to collaborate with Drs. Tim Kennedy and Susan Proudfoot, Directors of the Colorado Lung SPORE Lung Screening and Tissue Procurement Core II and the Lung Cancer Institute of Colorado; Dr. Wilbur Franklin, director of the Colorado Lung SPORE Tissue Procurement Core I; Dr. Basil Rigas Acting Chairman of the Division of Gastroenterology, New York Hospital-Cornell Medical Center, New York; and Dr. John Kovach, MD, Chairman of the Division of Oncology and his associate, Dr. Steven Sommer, MD, Ph.D., Professor of Molecular Biology at Mayo Clinic. (Please see letters of collaboration). Drs. Kennedy, Proudfoot and Franklin will provide lung tumors, bronchoscopy biopsy specimens, mucosal biopsy specimens, bronchial brushings, sputa and blood samples. Dr. Wilson has a pilot project funded by the SPORE Program. Five samples of sputum in 50 ml conical tubes have been received in Dr. Wilson's laboratory and have yielded adequate amounts of DNA for experiments. Dr. Rigas has offered to provide colon specimens including 85 carcinomas with corresponding normal tissue, 35 sporadic adenomas with corresponding normal tissue, 11 colonic samples from familial polyposis cases, and normal colon biopsies from 60 normal individuals. Drs. Kovach and Summers have offered to provide us with touch preps and amplified DNA from tumors with various defined p53 mutations.

## I BASIC RESEARCH

(i) **Development of a polymerase chain reaction/ligase chain reaction (PCR/LCR) method for identifying mutations in tumors.** This laboratory has used a combined PCR/LCR method to identify a K-ras mutation in colon polyps. As a first step to generalizing this technology, we will expand the number of simultaneous assays to identify mutations in codons 12, 13, and 61 of the K-ras gene and nine of the most common p53 gene mutations that occur in lung and colon cancers. Using cell lines and "touch prep" DNA samples we will develop the conditions that allow us to multiplex these reactions. Our initial aim is to be able to detect these mutations in lung and colon cancers. PCR/LCR will be compared to using LCR alone to see which technique achieves our target sensitivity of detecting one cancer mutation in  $10^2$  to  $10^3$  normal cells. PCR/LCR will also be compared to the PCR/LDR technique used in Project 2. The technique will be used to explore the frequency of mutations in lung and colon cancers. The mutations found will be correlated with the clinical parameters of the patients' conditions. Oligonucleotide or peptide nucleic acid addressable arrays (See Project 5) will let us expand the technology to screen for dozens or hundreds of mutations simultaneously.

(a) *Multiplex PCR/LCR detection of mutations in K-ras codons 12, 13, and 61.* The K-ras gene presents two significant challenges for mutation detection techniques. Extensive sequence homology between the H-, N- and K-ras genes makes relying on allele-specific PCR an uncertain and complicated prospect. Allele-specific PCR is further complicated by the proximity of the potential codon 12 and codon 13 mutations.



**PCR/LCR**

Gene Pos Codon

AAA ————— A  
 AA ————— C  
 A ————— T

K-ras 12 GGT 5' ————— GGT ————— 3'  
 3' ————— CCA ————— 5'

————— A ————— A  
 ————— C ————— AA  
 ————— T ————— AAA

AAA ————— A  
 AA ————— C  
 A ————— T

K-ras 13 GGC 5' ————— GGC ————— 3'  
 3' ————— CCG ————— 5'

————— A ————— A  
 ————— C ————— AA  
 ————— T ————— AAA

AAA ————— G  
 AA ————— C  
 A ————— T

K-ras 61 CAA

5' ————— CAA ————— 3'  
 3' ————— GTT ————— 5'

————— A ————— A  
 ————— G ————— AA  
 ————— C ————— AAA

Fig. 7. Scheme for PCR/LCR detection of mutations in codons 12, 13, and 61 of K-ras. (See following page for legend.)

Fig. 7. (See previous page). Scheme for PCR/LCR detection of mutations in codons 12, 13, and 61 of K-ras. At the top of the drawing is a schematic representation of the chromosomal DNA containing the K-ras gene. Exons are shaded and the position of codons 12, 13, and 61 shown. Allele specific primers are used to amplify only K-ras DNA flanking these three codons. The middle and bottom of the diagram gives a schematic representation of primer design for LCR detection of mutations in the second base of each codon. For example, codon 12 (GGT) may mutate to GAT, GCT, or GTT. Allele specific LCR primers contain the discriminating base on the 3' end and a "poly A" tail on the 5' end. Different mutations are distinguished by separating the products on a polyacrylamide gel. The drawing abbreviates the poly A tail for clarity. In the actual experiment, tails will differ from each other by two bases. The primers are designed such that they have a single 3' overhang with respect to each other. This primer design minimizes target independent amplification [60,61]. Note that LCR primers used for detecting mutations at codon 12 may interfere with hybridization of primers used to detect mutations at codon 13. However, primers for codon 13 cannot serve as "bridging" primers since they do not contain the codon 12 mutant sequence. It will need to be experimentally determined if these primers can correctly identify mutant signal in the presence of the other LCR primers. Additional sets of LCR primers would be required for detecting mutations in the first base of each codon, as well as the third base of codon 61 (not shown).

The scheme for simultaneously assaying codons 12, 13, and 61 of the K-ras gene for mutations is shown in Fig. 7. Carefully chosen PCR primer pairs or nested primer pairs will be used to PCR amplify (primarily) the K-ras gene in the regions surrounding codons 12 and 13 and codon 61. LCR primers will be synthesized for mutant sequences at the three codons. The LCR primers will have discriminating bases at their 3' ends. The Oligo 4.0 software program will be used to choose primers with  $T_m$  values of about 65°C. The common oligonucleotides will be synthesized with progressively longer 3' poly-A or hexaethylene oxide "tails" so the LCR products can be separated on sequencing gels. The 5' end of the discriminating primer will be labeled with  $^{32}\text{P}$ . After multiplex PCR amplification of the codon 12 and 13 region and the codon 61 region of K-ras, a multiplex LCR will be performed. LCR products will be separated by gel electrophoresis and detected by autoradiography. If multiplex LCR proves problematic, multiplex LDR detection would be used (see below and Project 2.)

PCR/LDR has been successfully applied to multiplex detection of cystic fibrosis [62-64], and 21 hydroxylase deficiency (D. Day, P. White and F. Barany, unpublished results). In the 21 hydroxylase study, individuals were determined to be heterozygous or homozygous for any of the ten common gene conversions that cause that disease. Likewise, PCR/LDR multiplexing has been used to distinguish between 30 different cystic fibrosis mutations, even when mutations were separated by only a single codon [64]. PCR/LCR has been successfully applied to multiplex detection of hyperkalemic periodic paralysis, where two sets of primers were partially overlapping [65]. It will have to be determined whether PCR/LCR can be multiplexed for detecting the mutations at codons 12 and 13 of K-ras. We presume that primers for the wild type sequences at these sites could act as "bridging" templates for each other. However, by using LCR primers only for the mutation sequences at these codons, bridging should not occur. As primer sets are verified, they will be added to a panel of mutation-detecting assays. A full complement of LCR primer sets should be able to detect all 20 possible mutations. It may be necessary to perform the LCR multiplexing in two sets: (a) Primers which detect mutations in the first bases of codons 12, 13, 61, as well as the last base of codon 61, and (b) Primers which detect mutations in the second bases of codons 12, 13, 61. This might minimize interference between the different primers. Should the presence of bottom strand primers interfere with proper LCR detection, the experiment could be performed using only the top strand primers for LDR detection. If needed, the LCR or LDR reactions could be performed in four tubes such that the primers did not interfere or overlap: (a) Primers for the first base of codons 12 and 61. (b) Primers for second base of codons 12 and 61, (c) Primers for first base of codon 13 and the third base of codon 61, and (d) Primers for second base of codons 13. Given the promising results with detection of mutations in neighboring codons of the cystic fibrosis gene using PCR/LDR [64], we are hopeful that experiments will be successful with the K-ras codon. Eventually the method will be tested using DNAs from a variety of cell lines. For codon 12 of the K-ras gene, mutant cell lines LS180 (colorectal adenocarcinoma, GGT → GAT) or A549 (lung carcinoma, GGT → AGT) or SK-CO-1 (colon adenocarcinoma, GGT → GTT) can be used. For codon 13 of the K-ras gene, LoVo cells (colorectal adenocarcinoma, GGC → GAC) can be used. All of these cell lines are available from the American Type Culture Collection repository.

(b) *Multiplex PCR/LCR for detection of 9 mutations in p53 exons 5, 6, 7 and 8.* The general strategy for simultaneous PCR/LCR detection of nine p53 gene mutations is shown in Fig. 8. Primer pairs or nested primer pairs will be used to amplify exons 5 & 6, 7, and 8 [82-84]. LCR primers will be synthesized for nine

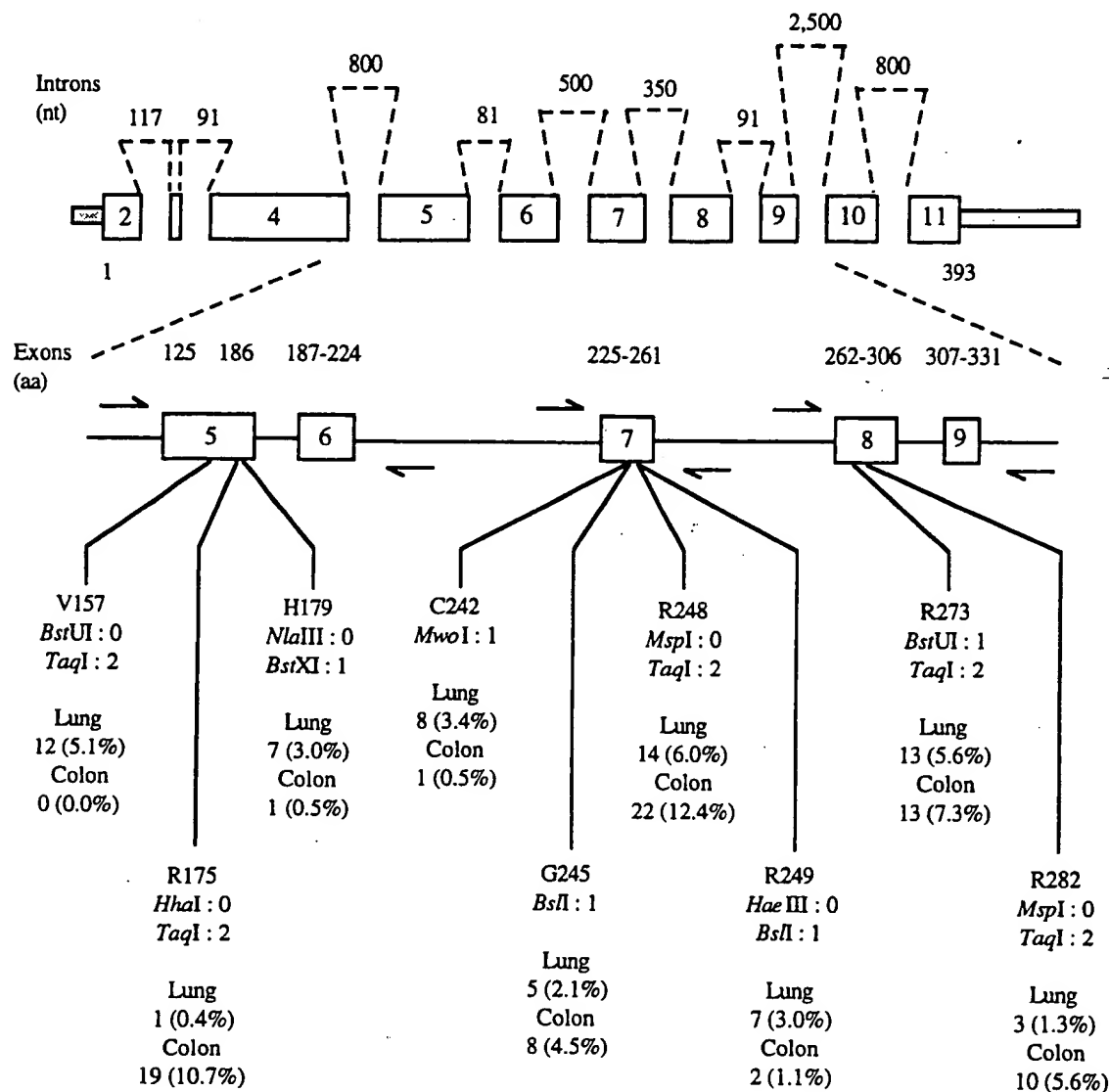


Fig. 8. Positions of the nine common p53 mutations in lung and colon cancer. This schematic of the p53 gene shows the approximate length and size of p53 exons and introns. Regions from exons 5 through 9 corresponding to amino acid residues 125 to 331 are shown in an expanded view. More than 95% of p53 point mutations have been found in these exons [28,29]. Horizontal arrows flanking the exons represent PCR primers (or nested primers if needed) for initial amplification of exons 5 & 6, 7, and 8. The positions of the nine common mutations are indicated, with the restriction site coded by the sequence : number of primers needed to convert to that site written below. For example, codon 248 is a natural *Msp*I site, and requires no conversion primers for that restriction endonuclease selection. The same codon would require two conversion primers to create a *Taq*I site. (See below and Project 2 for more details.) The number of mutations detected, and the percentage of total lung and colon mutations is shown below that codon. This represents 30% of 233 lung and 43% of 177 colon reported p53 mutations [28, 29]. Initially, LCR primer sets will be designed and synthesized for codons V157, R175, H179, R248, R249, and R282. These positions already contain a natural restriction site, and may be used immediately to test both PCR/LCR and PCR/RE/LCR detection of 1 mutation per  $10^5$  to  $10^7$  cells. The initial LCR primer set will be expanded to include all nine codons shown in this figure.

of the most common p53 mutations reported in lung and colon tumors; V157, R175, H179, C242, G245, R248, R249, R273, and R282 (see Fig. 9) [28, 29]. These codons account for approximately 30 percent of lung tumors and 43 percent of colon tumors p53 mutations isolated. The codons were chosen also because they represent restriction enzyme recognition sites or because they can be converted into restriction enzyme sites using one or two converting PCR primers. The ability to cleave at these codons makes these sites useful for PCR/RE/LCR (See section ii below). Those codons that can be cleaved without the use of converting

primers will be immediately useful for PCR/RE/LCR. Each LCR primer set will consist of two different length oligonucleotides with discriminating bases on the 3' ends. When more than one mutation has been reported for a codon, such as codon 248, additional primers with the discriminating base on the 3' end will be used. Ligation of one of these discriminating oligonucleotides to an adjacent common oligonucleotide will generate a product corresponding to the mutant allele. LCR primers will be designed to have a  $T_m$  of about 65°C with the aid of Oligo 4.0 software. Discriminating primers will be labeled with  $^{32}\text{P}$  at their 5' ends. The common oligonucleotides will contain poly A or hexaethylene oxide "tails" of increasing length on their 3' ends. Products will be separated on DNA sequencing gels and detected by autoradiography [60, 61, 64]. Alternatively, when the addressable array is synthesized and tested (see Project 5), LCR primers will have "zip code" tails, and signals will be distinguished by the position of fluorescent signals on the array. Signals would be quantified using a Molecular Dynamics FluorImager (See Core A and B). Both positive and negative controls (cell lines with a mutation in a different position) will be included. Examples of positive controls for hotspot mutations in the human p53 gene include codon 248: SW837 (lung adenocarcinoma, CGG → TGG, as noted above), codon 249: BT-549 (breast, ductal carcinoma, AGG → AGC), and codon 273: SW480 (colon adenocarcinoma, CGT → CAT). All of these cell lines are available from the American Type Culture Collection repository.

Similar to the situation for codons 12 and 13 of the K-ras gene, we presume that primers for the wild type sequences at codons R175 and H175 as well as codons C242 through R249 could act as "bridging" templates for each other. Using LCR primers for only the mutation sequences at these codons, bridging should not occur, but this will need to be determined empirically. Again, should bridging be a problem, only the top strand primers would be used in an LDR reaction. As primer sets are verified, they will be added to a panel of mutation-detecting assays. Initially, DNA prepared from known cell lines or sequenced tumor samples will be used to prove the method's ability to detect a given mutation. As primer sets are validated, they will be added to the reaction mixture primer pool. Subsequently, this technique will be tested with unknown samples derived from touch preparations of lung and colon tumors, which are generally homogenous for p53 mutations [28, 29, 82, 83]. Some of these codons have also been identified in breast cancers, and experimental results and conditions will be closely shared between Projects 1 and 2.

(c) *Improving the sensitivity of PCR/LCR mutation detection and comparing to PCR/LDR and LCR alone.* One of the key issues in any mutation detection scheme is the level of sensitivity. DNA prepared from cell lines or "touch" preps tends to be homogeneous. Thus, after PCR, a clean mutant signal is observed by sequencing or SSCP. When a mutant signal is less than 25 percent of the total, it may no longer contrast with wild type sequence. LCR, however, can be directed to the detection of only mutant sequences. Observing signals from mutant DNA then depends on both oligonucleotide hybridization and the fidelity of *Tth* ligase. Initial experiments in the Program Project Director's laboratory demonstrated the exquisite fidelity of *Tth* ligase. The enzyme showed a 50 fold discrimination between an A:T match and a G:T mismatch, and greater than 500 fold between an A:T match and an A:A mismatch. [60]. To broadly characterize the mutation amplification and detection methods used in Projects 1 and 2, we will compare LCR, PCR/LCR and PCR/LDR. The sensitivities and specificities of each and the ability to multiplex the method for the simultaneous detection of various mutations will be investigated. Optimizing conditions and/or using mutant *Tth* ligases may increase the sensitivity and specificity of any of the methods in the 1 copy in  $10^2$  to  $10^3$  range. The development of mutant *Tth* ligases is discussed in greater detail in Project 4.

(d) *Develop assays for K-ras codons 12, 13 and 61 and the nine p53 mutations for lung and colon tumors.* When the assays for the K-ras mutations and the nine p53 assays are established we will combine them to perform multigene, multiplex assays on DNA extracted from the lung and colon cancer specimens available to us. Eighty-five colon cancer specimens and 35 colon polyps are available to us for study. Some mutations will be confirmed by DNA sequencing. The frequency of p53 and K-ras mutations in our samples will be checked with mutation frequencies that others have obtained [28, 29] to see if they are comparable. Previous investigations [2] would lead us to expect a fewer p53 mutations in the colon polyps, although the small number of specimens may make comparison difficult. Lung tumors, bronchogenic carcinomas in situ and biopsies of severe bronchial dysplasia will be similarly investigated. When mutations are discovered in primary tumors we will attempt to locate lymph nodes and other tissues from the patient, which could be investigated for micrometastases with PCR/RE/LCR (See below). We will also attempt to develop the

conditions for PCR/LCR on DNA extracted from formalin-fixed, paraffin-embedded specimens. This would dramatically expand the number of specimens available to us for study.

(ii) **Development of a polymerase chain reaction/restriction endonuclease/ligase chain reaction (PCR/RE/LCR) to detect cancer mutations in one cell per  $10^6$  or  $10^7$  normal cells.** By PCR amplifying a DNA segment and digesting normal sequences, mutations in restriction endonuclease recognition sites can be selectively amplified. This laboratory has successfully identified 10 *H-ras* mutation-bearing plasmids from a background of  $10^9$  wild type copies. In another experiment a detection sensitivity of  $10^7$  was achieved, when one cell bearing a mutation in the first base of codon 248 in the p53 gene was identified out of  $10^7$  normal cells. In both cases these mutations eliminated an *Msp* I restriction endonuclease site. By amplifying and repeatedly digesting wild type sequence, the mutation was selected. Mutation signal was finally detected using LCR. A high level of sensitivity will be necessary for identifying micrometastases or early, preneoplastic cells in the sputum, stool samples and other clinical specimens.

The PCR/RE/LCR technology that we propose here will be used to identify cancer mutations in a few cells on a background of normal cells. This may have immediate application to clinical situations when the genetic changes in a primary tumor have already been determined. Minimal residual disease, the existence of micrometastases to lymph nodes and bone marrow or microinvasive disease could all be assessed. As the genetic changes of the early stages of carcinogenesis are discovered, screening for the premalignant phases of some cancers (e.g. the adenomatous polyps that precede colon cancer) may become feasible with this technology. For research purposes PCR/RE/LCR technology will allow us explore the early stages of tumorigenesis, aging and possibly help determine the mutagenic effects of exposures to chemicals and other potentially harmful agents.

Before positive results from these procedures can be interpreted as detecting the presence of a cancer mutation, a basic question must be answered: What is the background rate of single base substitution mutations in oncogenic loci in human tissues? We have begun to answer this question by analyzing peripheral blood leukocyte DNA from seven subjects for the G  $\rightarrow$  T transversion at the second base of codon 12 in the *H-ras* gene. One individual out of seven provided a positive result at a detection level of between one in  $10^5$  and one in  $10^6$ . Another aliquot of DNA from this individual is being re analyzed to verify the presence of this mutation in his leukocytes. A better approximation of the frequency of this mutation will probably require PCR/*Msp*I/LCR analysis of 60  $\mu$ g DNA, providing a sensitivity of one cell in  $10^7$ , and analysis of a larger number of blood specimens.

(a) *Develop PCR/RE/LCR to detect cancer mutations in one cell per  $10^6$  or  $10^7$  normal cells.* The technical process consists of amplifying DNA surrounding a region of interest and removing the normal sequence by restriction enzyme digestion. Codons V157, R175, H179, R248, R249 and R282 of p53 are part of restriction enzyme recognition sequences and are immediately testable with the PCR/RE/LCR technique. Codons H179, C242, G245 and R273 of p53 are convertible to restriction endonuclease recognition sequences using one modified PCR primer (see Table 2). (See also Project 2). Several sites in p53 can be converted to *Taq* I sites with two converting primers: V157, R175, R248, R273 and R282. PCR/RE/LCR assays for these mutations will be established using mutation targets diluted into corresponding normal samples. Mutation targets available for these experiments consist of synthetic oligonucleotide templates, previously characterized, amplified DNA samples, as well as cultured cell lines and tissue samples. Similar experiments will be performed in Project 2. Primers, protocols and results will be shared and compared.

A number of technical details about the assays will need to be addressed. Optimal conditions for the specific reactions will be determined. By using the appropriate mixtures of template DNA or DNA extracted from mixtures of cultured cells we will work out multiplexing of reactions that use the same restriction endonuclease (e.g. codons R248 and R282 of p53, which are *Msp* I sites). If a mutation can be detected using no conversions, one conversion, or two conversions, the advantages and disadvantages of each will be explored.

Table 2.

Codon	Percent of detected p53 Mutations in:		Restriction Endonuclease Recognizing this Codon using:		
	Lung Ca	Colon Ca	Zero Converting Primers	One Converting Primer	Two Converting Primers
V157	5.1	0.0	<i>Bst</i> UI		<i>Taq</i> I
R175	0.4	10.7	<i>Hha</i> I		<i>Taq</i> I
H179	3.0	0.5	<i>Nla</i> III	<i>Bst</i> XI	
C242	3.4	0.5		<i>Mwo</i> I	
G245	2.1	4.5		<i>Bsl</i> I	
R248	6.0	12.4	<i>Msp</i> I		<i>Taq</i> I
R249	3.0	1.1	<i>Hae</i> III	<i>Bsl</i> I	
R273	5.6	7.3		<i>Bst</i> UI	<i>Taq</i> I
<u>R282</u>	<u>1.3</u>	<u>5.6</u>	<i>Msp</i> I		<i>Taq</i> I
TOTAL	29.9	42.6			

Table 2. The nine p53 codons which will be tested for mutations in lung and colon cancers using PCR/LCR and PCR/RE/LCR. The expected percent of total p53 mutations at each codon for lung and colon cancers is listed [28, 29]. The restriction endonucleases recognizing wild type sequence at each codon after amplification with zero, one or two converting PCR primers is listed. See text for explanation.

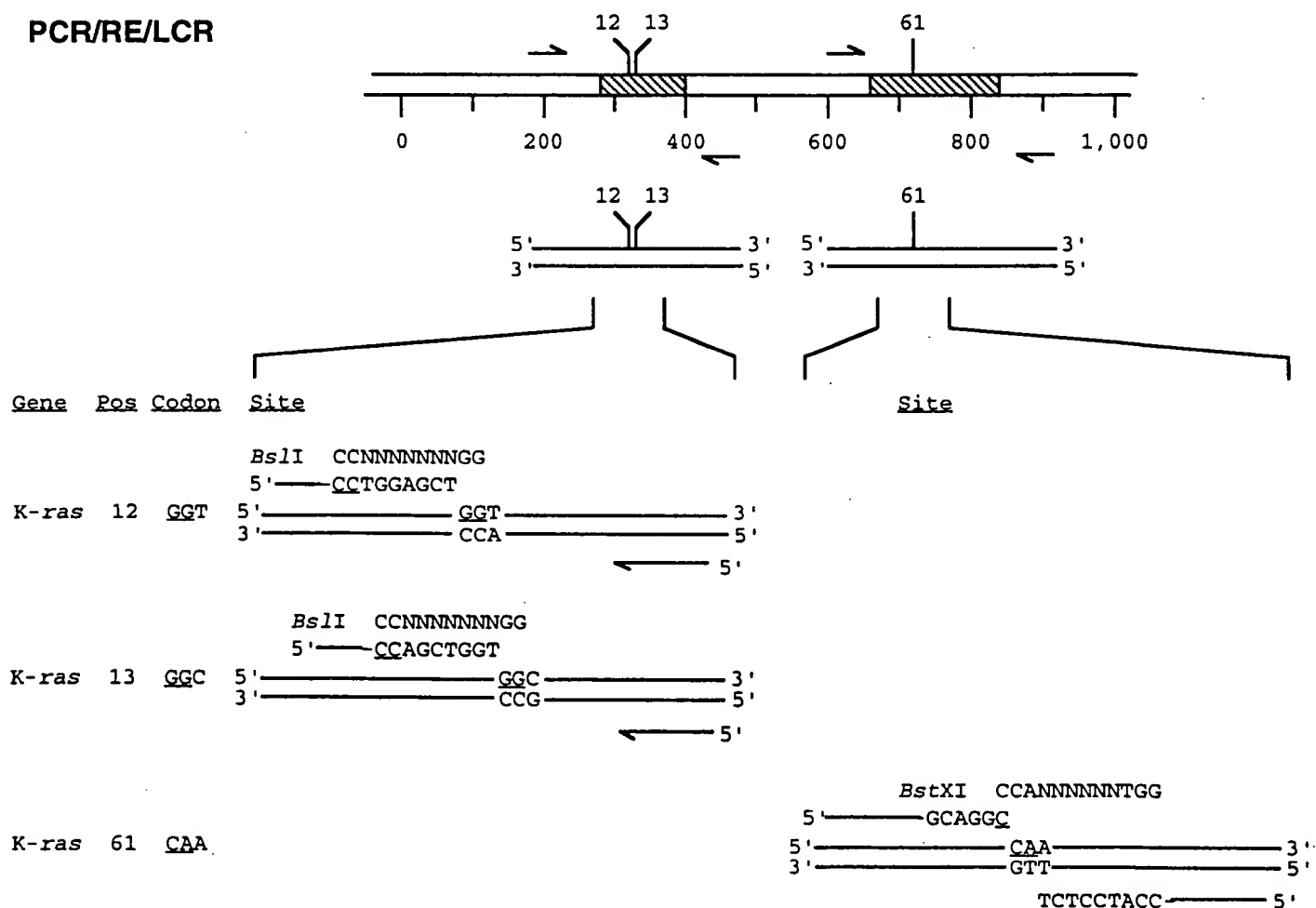
**PCR/RE/LCR**

Fig. 9. Schematic diagram of primers required for conversion of K-ras codons 12, 13, and 61 into restriction sites. See legend on following page.

Fig. 9. (See previous page.) Schematic diagram of primers required for conversion of K-ras codons 12, 13, and 61 into restriction sites. Top: Schematic representation of the chromosomal DNA containing the K-ras gene. Exons are shaded and the position of codons 12, 13, and 61 shown. Allele specific primers are used to amplify only K-ras DNA flanking these three codons. A second set of primers is used to convert the codon of interest into a restriction site. For codons 12 (and 13), one conversion primer and one outside primer are used to convert the sequence to a *Bst*I site. The conversion primer has two bases (CC) which mismatch to the target sequence, but since they are 7 and 8 bases from the 3' end, polymerase extension of these primers proceeds smoothly. The PCR reaction creates a *Bst*I site (CCNNNNNNNGG) at codon 12. After a first PCR amplification, the converted wild-type K-ras sequence may be cleaved by *Bst*I, while a mutation in either the first two bases (GGT) would be resistant to *Bst*I cleavage. A second round of PCR amplification would selectively amplify the mutant DNA. During the second round, a slightly nested outside primer would be used with the same conversion primer. Use of a nested primer avoids amplification of primer dimers or other unwanted amplicons. After three rounds of PCR amplification/*Bst*I selection, the mutant would be detected using the LCR primers depicted in Fig. 7. A different conversion primer (in a separate reaction) would be used for selectively amplifying mutations in codon 13. For codon 61, two conversion primers would be required for converting the wild-type K-ras sequence into a *Bst*XI site (CCANNNNNNTGG). In this scheme, one primer contains a mismatched base (C) on its 3' end, while the other has three mismatched bases (ACC) six bases from the 3' end. This scheme converts the wild type sequence into a *Bst*XI site, and would detect a mutation in the first two bases of codon 61 (CAA). (We recognize that the Gln codon could mutate into a His codon, and not be detected by *Bst*XI. However, Q61H mutations may be detected using a different set of conversion primers which generates a *Xmn*I site.) Although the first rounds of PCR amplification may be somewhat inefficient, once started, the amplification will proceed exponentially. The initial round of PCR amplification would be enhanced by use of nucleotide analogues "convertides" in the conversion primers (Project 3). Since this type of amplification does not allow for nested primers, a set of "zip code" containing primers would be used. For a more complete explanation of one and two sided restriction site conversions and the use of "zip code" primers, please see Project 2.

High sensitivity PCR/RE/LDR detection of mutant cells in K-ras codons 12, 13, and 61 will also require conversions to restriction sites (See Fig. 9). Codons 12 and 13 may both be converted to a *Bst*I site using one modified primer and a set of nested primers. These conversions would be done in separate reactions to avoid primer interference. Codon 61 may be converted to a *Bst*XI site. We are aware that the *Bst*XI conversion would not cover mutations in the third base of codon 61, however, conversion to *Xmn*I would cover that base. Conversion primers are shown in Fig. 10, and detection of mutant signal would be performed using the LCR primers shown in Fig. 7. The K-ras site conversion experiments could benefit from convertide nucleotide analogues (see Project 3), as well as an improved thermostable ligase (see Project 4). These K-ras conversion experiments will be done in collaboration with Project 2.

(b) *The presence of low level background mutations in tissue samples and base misincorporation by Taq polymerase.* The neoplastic transformation of a cell is caused by the cumulative effect of multiple mutations in growth-regulating genes. Presumably, many cells acquire mutations in many genes; cancers only arise from cells that have suffered mutations in specific combinations of genes. There are many questions that need to be answered about the rate of somatic mutations in cells: Does the rate of somatic mutation, at a specific site, increase with age? Do different tissues show different rates of mutation? Are exposures to carcinogens related to an increased rate of somatic mutation? To explore the background rate of mutations relevant to further investigations, we will examine normal biopsy tissues of lung and colon mucosa for the K-ras mutations and the p53 mutations found most commonly in tumors from those sites. This work must be done along with or prior to experiments looking at clinical specimens to detect early disease.

*Taq* polymerase is known to have a base misincorporation rate of approximately one nucleotide per  $10^6$  [78, 79]. Although this may vary for the various "read" and "write" combinations, starting with large numbers of templates ( $10^6$ - $10^7$ ) there is the possibility that misincorporation will lead to positive PCR/RE/LCR signal at some low rate. This may be especially true when a mismatched base is at the 3' end. Project 3 seeks to synthesize convertides which will ease the read step in the conversion process, and hopefully improve polymerase fidelity. It may also be possible that the presence of a 3' mismatch actually improves fidelity of incorporating the next base. Factors influencing polymerase fidelity will be more fully studied in Core B.

## II. CLINICAL RESEARCH

We have developed methodology for both low sensitivity (PCR/LCR) and high sensitivity (PCR/RE/LCR) mutation detection covering codons 12, 13, and 61 in K-ras and nine codons in the p53



tumor suppressor gene. These *k-ras* codons are mutated in approximately half of colon cancers. The nine codons account for approximately 43% of colon and 30% of lung p53 mutations. About half of lung and colon cancers have p53 mutations. We will screen tumor specimens for these mutations, and search other tissue (bone marrow, lymph nodes, blood) for the corresponding mutation as evidence of micrometastes. Clinical history and data will be available on all patients for which tissue specimens are obtained. We will correlate mutation information with all aspects of patients' histories and tumor histology. Important factors to consider will be age, family histories of cancer, smoking history, and other environmental exposures. We recognize that this constitutes an incomplete picture and represents only a pilot study.

(a) *Colon specimen procurement*: A spectrum of colonic tissue samples will be obtained through collaboration with Dr. Basil Rigas Acting Chairman of the Division of Gastroenterology, New York Hospital-Cornell Medical Center, and Dr. Gray Miller (Director, Tissue Procurement Core, University of Colorado Cancer Center). Stool, blood, colonic lavage samples, biopsies of normal appearing mucosa and samples of benign adenomas (when present), will be obtained at the time of colonoscopy in 4 cohorts of patients, including: 1) 40 subjects per year with no family history of colon cancer, no history of previous colonic neoplasia (adenoma or adenocarcinoma), and a normal colonoscopy; 2) 40 subjects per year with a family history of colon cancer, but no personal history of previous colonic neoplasia and a normal colonoscopy; 3) 40 subjects per year with current or previous colonic adenomas found at colonoscopy; and 4) Approximately 25 subjects with colon cancer. Tissue will be obtained both through the endoscopy suites of the New York Hospital-Cornell Medical Center and the University of Colorado Medical Center, and through the Tissue Procurement Core of the University of Colorado Cancer Center (see letters of collaboration from Dr. Gray Miller and Dr. Basil Rigas).

Tissues obtained from normal colonic mucosa, colonic adenomas, or carcinomas, obtained at the time of colonoscopic biopsy, or polypectomy, or at surgery, will be immediately frozen in liquid nitrogen and transported to Dr. Wilson's laboratory.

Colonic lavage samples in Denver will be obtained at the time of colonoscopy by suction of residual fluid from the colon during colonoscopy and by washing the colonic wall of the proximal mid and distal colonic segments with 0.9% saline via a jet washer and collecting the fluid by suction. These samples will be centrifuged (1500xg, 10 min), the pellet frozen in liquid nitrogen, and transported to Dr. Wilson's laboratory. Stool specimens will be collected by isolating a few grams of stool in 50 ml conical centrifuge tubes, frozen in liquid nitrogen, and transported on dry ice to Dr. Wilson's laboratory.

All of these procedures have been established and samples obtained as described above have been shown to yield adequate amounts of intact human DNA for the proposed studies. Consent forms for the harvesting of these tissues have been obtained.

(b) *Lung specimen procurement*. Lung tissue specimens will be obtained through the University of Colorado Lung Cancer SPORE Program (Dr. Wilson has a pilot project funded by the SPORE Program), and through collaborations with Dr. Tim Kennedy and Susan Proudfoot (Directors, Colorado Lung SPORE Lung Screening and Tissue Procurement Core II, and the Lung Cancer Institute of Colorado), and Dr. Wilbur Franklin (Director, Colorado Lung SPORE Tissue Bank Core I). Sputum, blood, and bronchoscopy biopsy specimens will be collected from: 1) 40 subjects per year with no definitive family history of cancer, no history of previous lung neoplasia, and a normal bronchoscopy; 2) 40 subjects per year with mild dysplasia found at bronchoscopy; 3) 40 subjects per year with moderate or severe dysplasia found at bronchoscopy; 4) Subjects with current carcinoma *in situ*; and 5) Approximately 40 subjects per year diagnosed with a lung carcinoma.

At present the SPORE Core units are accruing approximately 50 patients per month (~600 patients per year) who may be at risk of lung cancer due to job, age, smoking habits, and/or family history. It is estimated that approximately 210 patients with mild dysplasia, 100 with moderate or severe dysplasia, and 20 subjects with carcinoma *in situ*, will be biopsied and cytologically diagnosed per year. It is anticipated that blood, sputum, and bronchoscopy biopsy specimens will be obtained from between 10-15 patients where lung tumor tissue will also become available, per year. The SPORE Tissue Procurement Core II has already procured specimens from 220 subjects to date.



A subpopulation of 50 of these patients with moderate or severe dysplasia will be enrolled in a 13-cis-retinoic acid trial and multiple bronchoscopy biopsies will become available (at least two separate bronchoscopy procedures will be performed on this subset of patients at 6 month intervals).

All of these procedures have been established and samples obtained as described above have been shown to yield adequate quantities of DNA. Sputa specimens have been collected and stored in 50 ml conical tubes and five of these have thus far been transferred to Dr. Wilson's laboratory and have provided more than adequate amounts of intact DNA.

(c) *Testing K-ras codons 12, 13 and 61 and the nine p53 mutations on lung and colon tumor specimens using PCR/LCR.* When the assays for the K-ras mutations and the nine p53 assays are established we will combine them to perform multigene, multiplex assays on DNA extracted from the lung and colon cancer specimens available to us. Initially, 40 colon cancer specimens and 50 lung tumor specimens will be analyzed. Some mutations will be confirmed by SSCP and DNA sequencing, which are currently being performed in Dr. Wilson's laboratory. The frequency of p53 and K-ras mutations in our samples will be checked with mutation frequencies that others have obtained [28, 29] to see if they are comparable. When mutations are discovered in primary tumors we will attempt to locate lymph nodes and other tissues from the patient, which could be investigated for micrometastases with PCR/RE/LCR (See below).

(d) *Analysis of mutation rate in nontumor tissue specimens.* In order to interpret results from our high sensitivity technique, it will be necessary to determine the background mutation rate in normal tissue. The detection of mutation rates at a given codon within non-tumor tissue specimens will require techniques that can detect one mutant in  $10^6$  or more cells. PCR/MspI/LCR assays described above are already appropriate for looking at background mutation rates. We will look at a dozen each, of DNA isolated from normal colon and lung tissue, as well as blood, sputum, and stool samples to determine the background mutation rate. This will be compared with positive controls consisting of mixtures of mutant in normal DNA from known tissue cell cultures.

(e) *Identification of Micrometastases.* With this sensitive PCR/RE/LCR assay, the presence of micrometastases will be evaluated in individual cancer cases. Once specific mutations have been identified using PCR/LCR on the tumor, lymph nodes, blood, and adjacent resected tissue can be analyzed by these PCR/RE/LCR techniques. These tissues will also be examined for the presence of micrometastases by normal histological or immunohistochemical analysis. Results of these standard techniques will be compared to those obtained by the DNA diagnostic PCR/RE/LCR method described here. We expect approximately 8 colon and 8 lung tumors to have one of the nine p53 mutations detectable by our PCR/LCR assay. This is a pilot investigation into the feasibility of performing these high sensitivity mutation detection studies. We do not necessarily expect to find micrometastases by DNA methods in early stage cancers, given our sample size. We do expect to detect lymph node metastases by our DNA methods if they are also detectable by the standard histological or immunohistochemical analysis. Blood specimens from those patients with lymph node metastases will also be analyzed. It is possible that blood, sputum, or colonic lavage or stool samples may also contain cells carrying the tumor mutation. We are aware of the possibility that this presents for early detection of cancers [66]. We will explore the possibility of analyzing these specimens by PCR/RE/LCR for early detection of cancer. Larger scale studies will be needed to access the ultimate prognostic and diagnostic utility of this technique.

### III. PATIENT POPULATION AND ELIGIBILITY

The composition of the patient population included in this study will depend on the oncology patient population available at The Children's Hospital, the Lung Institute of Colorado, the University of Colorado Health Sciences Center Hospital, and The New York Hospital-Cornell Medical Center. Although every effort will be made to increase the proportion of females and minorities, the available population has in the past been predominantly male Caucasian. Both the Lung Tumor Bank and the Sputa Bank for the Lung Cancer SPORE grant here at UCHSC, contain predominantly white male specimens. However, these tissue banks have only been collecting specimens for 6 months and it is anticipated the minority representation, especially female and

hispanic, will be increased significantly as the collection of specimens moves to more outlying clinics. As to childhood cancers, in 1988, the distribution of oncology patients at The Children's Hospital was: 68% male; 35% female, 82% white, 2% black, 15% Hispanic, 0% oriental, and 1% American Indian.

All patients entered into this project must fit the following criteria:

- 1) Be diagnosed with cancer, and/or be entered in a clinical study (therefore consent)
- 2) Availability of clinical history
- 3) Availability of family history

#### IV. GENERAL LABORATORY METHODS

(a) *Blood Samples and Tissue Specimens.* Approximately 10-20 ml of blood will be drawn in EDTA vacutainer tubes, and sent to the laboratory. The nucleated cells will be separated by centrifugation as the buffy coat as quickly as possible after the sample is drawn from the patient. DNA will then be isolated by standard methods as described previously [85]. (These procedures are routine in Dr. Wilson's laboratory which provides clinical laboratory testing services.) Normally a minimum of 100 µg of DNA (generally between 100 µg and 300 µg) can be isolated from 5 ml of blood.

Tissue specimens will be obtained from surgery and the DNA isolated immediately or snap frozen in liquid nitrogen and stored at -70°C. Tissue samples of approximately 5 x 5 x 5 mm size will be washed extensively in Hepes Buffered Saline followed by gently homogenization in RBC lysis buffer (0.144 M NH<sub>4</sub>Cl and 0.01 M NH<sub>4</sub>CO<sub>3</sub>, pH 7.4). The cells will be pelleted by centrifugation and the process repeated until the pellet has a pale white appearance. The cells will then be lysed and the DNA isolated as described above.

Cell pellets isolated from sputa, bronchiolar lavage, or colorectal washes will be handled the same as surgical tissue specimens. The isolation of DNA from mucosal cells in the feces requires some modifications and has been performed in this laboratory by following the protocol used in the laboratories of Dr. Bert Vogelstein and Dr. Curt Harris. Briefly, approximately 1 g of frozen (-70°C) stool is placed in 6 ml of a solution of 500 mM Tris, 16 mM EDTA, 10 mM NaCl, pH 9.0, and vortexed and centrifuged to remove particles and bacteria. The solution is then treated with proteinase K and 1% SDS and incubated at 60°C, prior to phenol and chloroform extraction (as described above). The DNA is pelleted by centrifugation upon the addition of the ethanol, resuspended in TE buffer and the human DNA purified in an FMC spin Bind™ DNA extraction column prior to analysis.

(b) *Tissue Cultures.* Human cell lines will be obtained from the American Type Culture Collection (ATCC) or the NIGMS Human Genetic Mutant Cell Repository and propagated by standard methods [86, 87], as either surface or suspension cultures in the recommended media and percentage of FBS and growth factors as required. (These procedures are routinely performed in the clinical service laboratory of Molecular Genetics/Oncology under the direction of Dr. Wilson here at The Children's Hospital.)

(c) *Mutant Selection (PCR/MspI/LCR).* These procedures have been reported in abstract form by us and follow the same basic method as reported by Felley-Bosco et al. [41], are described briefly here. Either 6 or 60 µg of genomic DNA is restricted overnight with *MspI* (or other appropriate restriction enzyme) in the manufacturer's recommended buffer in a minimum volume. The whole restricted solution is subjected to PCR amplification for 20-25 cycles, followed by a five hour *MspI* restriction of an aliquot from this amplification solution. An aliquot of this second *MspI* restriction solution is then subjected to 20 cycles of nested PCR amplification, and the five hour *MspI* restriction of a portion of this amplification solution repeated as before. A third cycle of internally nested PCR using a third set of primers, is performed for 20 cycles along with the subsequent five hour *MspI* restriction. This selection process contains a total of three PCR amplification (totaling 65 cycles) and four *MspI* restriction procedures prior to LCR analysis.

The above PCR amplifications have been performed using *Taq* polymerase. Should the mutation frequency of any locus studied be found to be higher than that observed in *H-ras* codon 12 or p53 codon 248 (as noted in the Preliminary Studies), then other thermostable polymerases with proofreading activity from

either *Thermatoga maritima* [88], *Thermococcus litoralis* [89], or *Pyrococcus species GB-D* [90] will be tested. (Please see letters of collaboration from Dr. David Gelfand, Roche Molecular Systems, and Drs. Richard Roberts, Ira Schildkraut and Geoffrey Wilson of New England Biolabs Inc.). Results with the proofreading polymerases ("Tma", "Vent", and "Deep Vent") would be compared to the non-proofreading polymerases ("Taq", "Tth", "Vent exo3-"). These polymerase fidelity experiments will be done in collaboration with Project 2 and Core B.

(d). *LCR and Detection*. These procedures have been well described by Dr. Barany [60, 61]. Briefly, one  $\mu$ l of the PCR/MspI selection solution is subjected to 30 cycles of LCR (or LDR) amplification and detection in the presence of 200,000 cpm of  $^{32}$ P end-labeled invariant primers, 1  $\mu$ M of each wild type or each mutant discriminating primer (or a mixture of all primers, each at 1  $\mu$ M concentration), 4  $\mu$ g Salmon sperm DNA, 0.1% triton X-100, and 10 U Taq ligase in standard LCR buffer. The amplification process is a two cycle reaction using 94°C for denaturation and 65°C for ligation. The resulting ligation products are resolved on a 7 M urea, 10% acrylamide sequencing gel. The gel is dried and exposed to X-ray film.

#### IV. STATISTICAL ANALYSES

Since each study will require several analyses, such as mutational spectra as well as biological issues, the general statistical capability will be described first. The data will be entered into a database (e.g. Paradox), verified, and transferred to other programs as required. SYSTAT and SAS are the primary statistical and graphics packages available at this institution. SYSTAT is currently available both on Apple MacIntosh and 486 IBM compatible systems. SAS for Windows is available on 486 IBM compatible systems.

The search for mutation patterns for various tissues and tumors will be primarily graphical using stars, Chernoff's faces, etc. Various forms of cluster analysis and logistic regression may be employed. Mutations frequencies will be calculated using Poisson models. Logistic regression will be used with clinical and genetic information to predict tumor development associated mutations. Once logistic models are derived, they will be tested on a new set of subjects and specimens. Thus, logistical regression analyses will be used to categorize individuals, tumors, or specific tissues, based on the results of mutational analyses of multiple oncogenic loci. The actual handling of the results will vary for different portions of these studies. Determining the population average background mutation frequencies for each site may require an unreasonable number of individuals, while the analyses of background spectra may be more reasonable. Multiple samplings from individuals will also alter the statistical analysis.

Biostatistical consultation services are also available through the UCHSC Cancer Center.

#### E. PROGRAM ASPECTS

This project presents two technologies for the detection of point mutations in cancers. Based on DNA amplification techniques that combine sensitivity and specificity, their feasibility will be demonstrated by investigating mutations in colon and lung cancers; and exploring the natural background rate of somatic mutations at specific loci in human DNA. The project will include (i) Development of a polymerase chain reaction/ligase chain reaction (PCR/LCR) multi-gene, multi-mutation detection system to simultaneously identify mutations in three codons of the *k-ras* oncogene and nine codons of the *p53* tumor suppressor gene. When they are fully developed PCR/LCR should be able to detect tens to hundreds of mutations at a sensitivity of one in  $10^2$  or  $10^3$ . Approximately half of colon cancers have these *k-ras* mutations. About 15 percent of lung tumors and about 21 percent of colon cancers have one of these nine *p53* mutations. Using PCR/LCR to identify these mutations we will investigate 40 colon and 50 lung tumors. (ii) Refine the polymerase chain reaction/restriction endonuclease digestion/ligase chain reaction (PCR/RE/LCR) to detect the above *p53* mutations at sensitivities of one in  $10^7$ . PCR/RE/LCR selectively amplifies a mutation sequence by removing normal sequence. PCR/RE/LCR has already detected one mutation-bearing cell out of  $10^7$  normal cells. We will first use PCR/RE/LCR to determine the natural background mutation rate in non-cancerous tissues. Then, for patients whose tumors had detectable *p53* mutations, we will use PCR/RE/LCR to investigate lymph nodes, blood and bone marrow specimens for micrometastases.

Project 1 and Project 2 are working jointly to develop the gene amplification and mutation detection methods that are the heart of this Program Project. Two projects, however, will explore slightly different variations on the same methods, each of which may have its particular advantages. Achievements by one group will be immediately transferable to the other. Meanwhile, Project 3 will help improve and expand the PCR/RE/LCR technique by providing nucleotide analogues that will serve as convertides and universal base pairs allowing more mutations to be analyzed. An improved thermostable ligase, the aim of Project 4, may increase the specificity of these ligase-based detection techniques. The ultimate goal of this Program, being able to detect hundreds of mutations simultaneously, will be achieved finally with the advent of addressable arrays, produced by Project 5. Informatic support (Core A) will be crucial for Project 1, designing primers, keeping track of assay conditions, and recording results for analysis. Core B, instrumentation, will supply oligonucleotides for all the PCR/LCR and PCR/RE/LCR assays performed by Project 1.

The exploration of the *k-ras* and *p53* gene mutations that will be carried out in Project 1 (and Project 2) is only the beginning of efforts to examine tumors for multiple mutations and detect small foci of cancer cells by their genetic signatures. A host of known genes such as APC, MSH2, NF1, NF2, WT1, DCC, MCC, VHL, *ret*, and the *ras* genes play significant roles in various cancers. Hotspots have recently been reported for the *ret*, APC and VHL genes (91-93). These and other genes that will be discovered during the existence of this Program (e.g. BRCA1) will undoubtedly be candidates for correlations between mutations and cancer outcomes. The ability to simultaneously detect a variety of mutations at high sensitivities has been of great interest to several of our collaborators. Dr. Timothy Kennedy is working on advancing the clinical care and early diagnosis of lung cancer. Drs. Gary Miller and Basil Rigas are interested in *ras* and other genes mutated in colon cancer (and prostate cancer, Dr. Miller). Dr. John Sninsky is also interested in *ras*, but in the context of pancreatic cancer. Dr. Thierry Soussi has studied cancers of many varieties and made much of his data available to us. Please see letters of collaboration in the overview section of this program project grant.

## F. TIMETABLE

### Task 1. Developing a polymerase chain reaction/ligase chain reaction (PCR/LCR) method for identifying mutations in tumors.

- a. Designing and synthesizing PCR and LCR primers for *K-ras* codons 12, 13, and 61, and for nine *p53* mutations for lung and colon cancers. Testing these primers on cell lines and tumor specimens with known mutations. Months 1-18
- b. Developing a multiplex PCR/LCR assay to simultaneously detect *K-ras* codons 12, 13, and 61, and nine *p53* mutations for lung and colon cancers. Optimizing the procedure to detect 1 mutation in  $10^2$  to  $10^3$  normal cells. Exploring the difference between LCR only, PCR/LCR, and PCR/LDR. Comparing results with Project 2. Months 18-36.

### Task 2. Developing a polymerase chain reaction/restriction endonuclease/ligase chain reaction (PCR/RE/LCR) to detect cancer mutations in one cell per $10^6$ or $10^7$ normal cells.

- a. Developing PCR/RE/LCR for *K-ras* codons 12, 13, and 61, and for nine *p53* mutations for lung and colon cancers. Comparing results between *p53* mutation detection with PCR/RE/LCR at natural sites (V157, *Bst*UI; R175, *Hha*I; R248, *Msp*I; and R282, *Msp*I) with two sided conversion (to *Taq*I sites). Comparing these results with 1 sided conversions (H179, *Bst*XI; C242, *Mwo*I; G245, *Bst*II; R249, *Bst*II; and R273, *Bst*UI). Months 13-36
- b. Exploring the natural mutation rate and possibility of polymerase misincorporation using the high sensitivity technique. The natural mutation rate will be explored in normal tissues from healthy individuals and the misincorporation rate of the polymerase will be investigated using synthetic oligonucleotides. (In collaboration with Core B and Project 2.) Months 12-36
- c. Characterizing mutations in lung and colon cancers beginning with the *K-ras* mutations and the first 9 *p53* mutations and adding other assays as they are developed. Months 13-60
- d. Testing the ability of PCR/RE/LCR to detect low levels of cancer mutations in clinical specimens. The ability of this method to detect micrometastases will be explored. The early diagnosis of cancers will be

explored. Sputa, bronchialveolar lavage samples, stool and colorectal washings will be examined. Months 24-60

## G. HUMAN SUBJECTS

1. The composition of the patient population included in this study will depend on the oncology patient population available at The Children's Hospital, the Lung Institute of Colorado, the University of Colorado Health Sciences Center Hospital, and the New York Hospital-Cornell Medical Center. Although every effort will be made to increase the proportion of females and minorities, the available population has in the past been predominantly male Caucasian. Both the UCHSC Cancer Center Tissue Bank, the Lung Tissue Bank and the Sputa Bank for the Lung Cancer SPORE grant here at UCHSC, contain predominantly white male specimens. However, these Lung tissue banks have only been collecting specimens for 6 months and it is anticipated the minority representation, especially female and hispanic, will be increased significantly as the collection of specimens moves to more outlying clinics. As to childhood cancers, in 1988, the distribution of oncology patients at The Children's Hospital was: 68% male; 35% female, 82% white, 2% black, 15% Hispanic, 0% oriental, and 1% American Indian.

All patients entered into this project must fit the following criteria:

- 1) Be diagnosed with cancer, and/or be entered in a clinical study (therefore consent)
- 2) Availability of clinical history
- 3) Availability of family history

2. All patient related materials including clinical records, as well as biological samples (blood, sputa, bronchial brushings and biopsies, bronchial lavage, stool, polyps and mucosal colonoscopy, tumors and normal adjacent resected tissues) will be used for the research purposes described in this grant and these materials will not be used for any other purpose. Small blood samples will be obtained from this patients enrolled in SPORE programs and in the UCHSC Cancer Center Colon Cancer programs, as well as the Hereditary Cancer Clinic. Tumor tissue and, when available, adjacent normal tissue specimens will be obtained following routine clinically indicated surgery and/or at autopsy.

3. Subjects will be recruited jointly by members of the UCHSC Cancer Center Lung Cancer SPORE Program, UCHSC Cancer Center and by members of Oncology Department of The Children's Hospital. An institution IRB approved consent form will be discussed with the eligible patient, and families members when applicable, by the attending physician. The nature of the study, the drawing of blood and tissues to be biopsied, and the possible problems will be discussed in detail with the patient and family.

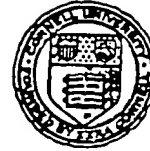
4. The potential risks of this study to the patient are small, amount to the discomfort and slight increase in risk of infection related to drawing blood from an indwelling catheter or from a venous puncture, or from tissue biopsies taken from the bronchial mucosa or colorectal mucosa.

5. In order to minimize risk of infection, blood samples will be drawn by oncology staff nurses and physicians trained in proper sterile technique for handling indwelling central venous lines, bronchoscopy, and colonoscopy procedures. Institutional procedure policies will be followed. These are procedures commonly performed at the respective institutes and clinics and are likely to have minimal risks. Signs and symptoms of infection will be explained to participants and they will be assigned an identification number. The patient's name will not be used. Results of these tests will be considered experimental until the study has been completed and analyzed. Therefore, the results will not be made available to the patient, parent, or health care providers not directly involved in this research program. Since no data presently exists to suggest clinical decisions should be based on these studies, there will be no problem with confidentiality of these results.

**H. CONSULTANTS/COLLABORATORS:** Letters and Biographical Sketches for collaborators are attached in the overview section of this program project grant.

**I. CONSORTIUM/CONTRACTUAL ARRANGEMENTS:** Please see following page.

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**STATEMENT OF INTENT TO ESTABLISH A CONSORTIUM AGREEMENT**

**Date:** January 26, 1994

**Grant Number:** P01-

**P-01 Application Title:** PROGRAM PROJECT: NEW METHODS FOR  
 CANCER DETECTION

**Project # 1; GENETIC MARKERS OF LUNG AND  
 COLON CANCER.**

**Proposed Project Period:** Year 01; 12/01/94- 11/30/95

"The appropriate programmatic and administrative personnel of each institution involved in this grant application are aware of the NIH consortium grant policy and will establish the necessary inter-institutional agreement(s) consistent with that policy."

**CORNELL UNIVERSITY MEDICAL  
 COLLEGE, NEW YORK, NY**

(Applicant Institution)

**CHILDRENS HOSPITAL  
 DENVER, CO.**

(Consortium Institution)

\_\_\_\_\_  
 (name) (date)  
**Principal Investigator:**

**FRANCIS BARANY, Ph.D.**

\_\_\_\_\_  
 (name) (date)  
**Co-Investigator:**

**VINCENT L. WILSON, Ph.D.**

\_\_\_\_\_  
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**Official Authorized to Sign for Institution**

**GREGORY W. SISKIND, M.D.  
 ASSOCIATE DEAN**

\_\_\_\_\_  
 (name) (date)  
**Official Authorized to Sign for Institution**

**J. LITERATURE CITED.**

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## **Project 2.**

### **Genetic Markers of Breast and Cervical Cancer**

**Project Leader: Francis Barany  
Cornell University Medical College**

**Project Co-Leader: Matthew Lubin  
Strang Cancer Prevention Center**

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. DO NOT EXCEED THE SPACE PROVIDED.

To improve cancer care, researchers and clinicians need robust methods of identifying genetic alterations in cancers. There are three important challenges that need to be met: (i) the detection of many possible point mutations in tumors; (ii) the quantification of gene amplifications and deletions in tumors; and (iii) the detection of rare cancer cells against a background of normal cells. Researchers need these capabilities to be able to correlate multiple genetic alterations with clinical outcomes, identify new cancer-related genetic loci, and detect early cancer recurrence and premalignant cell.

To accomplish this, we will develop: (i) a multiplex polymerase chain reaction/ligase detection reaction (PCR/LDR) system to detect many possible point mutations in cancers; (ii) a multiplex ligase detection reaction/polymerase chain reaction (LDR/PCR) system to quantify gene amplifications and deletions in tumors; and (iii) a polymerase chain reaction/restriction endonuclease/ligase detection reaction (PCR/RE/LDR) to identify 1 cancer cell in  $10^6$  normal cells.

Some issues in cancer will be explored. Specifically we will: (i) Expand PCR/LDR to detect 24-40 point mutations, (63% to 79% of p53 gene mutations) in breast tumors. PCR/LDR will also be used to detect high risk human papillomavirus (HPV) in cervical lavages or biopsies. (ii) Use "zip code" primers to proportionally PCR amplify and quantify LDR products of genes deleted or amplified in tumors. This should allow us to simultaneously detect HER-2/neu and int-2 gene amplifications, as well as p53 gene deletions in breast tumors. (iii) Refine PCR/RE/LDR for detecting five p53 mutations by selectively amplifying mutated DNA while removing wild-type products by *TaqI* restriction endonuclease cleavage. We will then characterize p53 gene mutations and deletions, HER-2/neu amplifications, and int-2 amplifications in 100-200 frozen breast tumors and corresponding fixed specimens. We will use PCR/RE/LDR to look for micrometastases in the bone marrow and lymph nodes of patients whose tumors had one of the five specific p53 mutations. Ultimately, this high sensitivity method may identify early relapses or primary tumors by detecting circulating cancer cells in the blood.

PERSONNEL ENGAGED ON PROJECT, INCLUDING CONSULTANTS/COLLABORATORS. Use continuation pages as needed to provide the required information in the format shown below on *all* individuals participating in the project.

Name	<u>BARANY, Francis</u>	Degree(s)	<u>Ph.D.</u>	Social Security #	<u>REDACTED</u>
Position Title	<u>Associate Professor</u>	D.O.B.	<u>REDACTED</u>	Role on Project	<u>Prin. Investig.</u>
Organization	<u>Cornell University Medical College</u>			Department	<u>Microbiology</u>
Name	<u>LUBIN, Matthew</u>	Degree(s)	<u>M.D.</u>	Social Security #	<u>REDACTED</u>
Position Title	<u>Director of Medical Genetics</u>	D.O.B.	<u>REDACTED</u>	Role on Project	<u>Co-investigator</u>
Organization	<u>Strang Cancer Prevention Center</u>			Department	<u>Medical Genetics</u>
Name	<u>DAY, Darren</u>	Degree(s)	<u>Ph.D.</u>	Social Security #	<u>REDACTED</u>
Position Title	<u>Research Associate</u>	D.O.B.	<u>REDACTED</u>	Role on Project	<u>REDACTED</u>
Organization	<u>Cornell University Medical College</u>			Department	<u>Microbiology</u>
Name	<u>KOLLER, Antje</u>	Degree(s)	<u>B.A. equiv.</u>	Social Security #	<u>REDACTED</u>
Position Title	<u>Technician</u>	D.O.B.	<u>REDACTED</u>	Role on Project	<u>REDACTED</u>
Organization	<u>Cornell University Medical College</u>			Department	<u>Microbiology</u>
Name		Degree(s)		Social Security #	
Position Title		D.O.B.		Role on Project	
Organization				Department	
Name		Degree(s)		Social Security #	
Position Title		D.O.B.		Role on Project	
Organization				Department	
Name		Degree(s)		Social Security #	
Position Title		D.O.B.		Role on Project	
Organization				Department	

DD

Principal Investigator/Program Director (Last, first, middle): **F. BARANY, Ph.D.**  
**DETAILED BUDGET FOR INITIAL BUDGET PERIOD**  
**DIRECT COSTS ONLY**

FROM 94/12/01 THROUGH 95/11/30

PERSONNEL (Applicant Organization Only)				DOLLAR AMOUNT REQUESTED (omit cents)			
NAME	ROLE ON PROJECT	TYPE APPT. (months)	% EFFORT ON PROJ.	INST. BASE SALARY	SALARY REQUESTED	FRINGE BENEFITS	TOTALS
Francis Barany	Principal Investigator	12	15				
Matthew Lubin	Co-Investigator	12	5				
Darren Day	Research Associate	12	100				
Antje Koller	Research Technician	12	15				
<b>PROJECT 2</b>							
<b>SUBTOTALS</b>					<b>\$64,288</b>	<b>\$20,572</b>	<b>\$84,860</b>
<b>CONSULTANT COSTS</b>							<b>\$0</b>
<b>EQUIPMENT (Itemize)</b>							<b>\$0</b>
<b>SUPPLIES (Itemize by category)</b>							<b>\$14,500</b>
Glassware & Plastic \$3,000							
DNA Modifying Enzyme: \$5,000							
Chemicals & Reagents \$6,500							
<b>TRAVEL</b>							<b>\$1,200</b>
One trip per year for P.I. to present results \$1,200							
<b>PATIENT CARE COSTS</b>							<b>\$0</b>
INPATIENT							
OUTPATIENT							<b>\$0</b>
<b>ALTERATIONS AND RENOVATIONS (Itemize by category)</b>							<b>\$0</b>
<b>OTHER EXPENSES (Itemize by category)</b>							<b>\$3,000</b>
Phone, Xerox, Page Char: \$3,000							
<b>SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD</b>							<b>\$103,560</b>
<b>CONSORTIUM/CONTRACTUAL COSTS</b>							
DIRECT COSTS \$0				TOTAL			<b>\$0</b>
INDIRECT COSTS \$0							
<b>TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD</b>							<b>\$103,560</b>

(Item 7a, Face Page)

**BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD  
DIRECT COSTS ONLY**

PROJECT 2

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED			
			2nd	3rd	4th	5th
<b>PERSONNEL:</b>						
<i>Salary &amp; fringe benefits</i>						
<i>Applicant organization only</i>		\$84,860	\$140,639	\$146,265	\$152,116	\$158,201
<b>CONSULTANT COSTS</b>		\$0	\$0	\$0	\$0	\$0
<b>EQUIPMENT</b>		\$0	\$2,000	\$2,000	\$2,000	\$2,000
<b>SUPPLIES</b>		\$14,500	\$21,080	\$21,923	\$22,800	\$23,712
<b>TRAVEL</b>		\$1,200	\$1,248	\$1,298	\$1,350	\$1,404
<b>PATIENT CARE COSTS</b>	<b>INPATIENT</b>	\$0	\$0	\$0	\$0	\$0
	<b>OUTPATIENT</b>	\$0	\$0	\$0	\$0	\$0
<b>ALTERATIONS AND RENOVATIONS</b>		\$0	\$0	\$0	\$0	\$0
<b>OTHER EXPENSES</b>		\$3,000	\$3,120	\$3,245	\$3,375	\$3,510
<b>SUBTOTAL DIRECT COSTS</b>		\$103,560	\$168,087	\$174,731	\$181,641	\$188,827
<b>CONSORTIUM/ CONTRACTUAL COSTS</b>		\$0	\$0	\$0	\$0	\$0
<b>TOTAL DIRECT COSTS</b>		\$103,560	\$168,087	\$174,731	\$181,641	\$188,827
<b>TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD</b>						<b>\$816,846</b>
						<i>(Item 8a)-&gt;</i>

## JUSTIFICATION (Use continuation pages if necessary):

**From Budget for Initial Period:** Describe the specific functions of the personnel, collaborators, and consultants and identify individuals with appointments that are less than full time for a specific period of the year, including VA appointments.

**For All Years:** Explain and justify purchase of major equipment, unusual supplies requests, patient care costs, alterations and renovations, tuition remission, and donor/volunteer costs.

**From Budget for Entire Period:** Identify with an asterisk (\*) on this page and justify any significant increase or decrease in any category over the initial budget period. Describe any change in effort of personnel.

**For Competing Continuation Applications:** Justify any significant increases or decreases in any category over the current level of support.

**Personnel:** Cornell University Medical College and Strang Cancer Prevention Center salaries are in accordance with the high cost of living in New York City, as well as the experience of the personnel. A 15% effort by the Principal Investigator and a 5% effort by Co-investigator assures full supervision of the junior personnel in this project. The Co-investigator will increase his effort and salary to 10% for years 2-5 reflecting increased responsibility in the project as our testing of breast biopsies expands. Cornell University Medical College has granted the Principal Investigator a Hirschl/Monique Weill-Caulier Career Scientist Award from 01/01/92 to 01/01/1997. This award of \$20,000 / year may be used as salary (and fringe benefit support) only. It thus allows the P.I. to spend full effort on research.

Dr. Darren Day is a research associate (Ph.D.) who has been in the Principal Investigator's laboratory since 10/10/92. Dr. Day has four years of postdoctoral experience and is a highly skilled member of the Principal Investigator's laboratory. He developed the PCR/LDR method for multiplex discrimination of ten



gene conversion mutations which cause 21 hydroxylase deficiency. In collaboration with the Principal Investigator, he has participated in the PCR/RE/LDR experiments. He has already designed the extensive set of primers required for detection of high risk HPV responsible for cervical carcinomas. We are also requesting a Postdoctoral Fellow for the Co-Investigator for years 2-5 to perform the PCR/LDR and LDR/PCR experiments on the 100-200 breast tumor samples. Ms. Antje Koller has been a highly skilled research technician in my laboratory for the past 8 years. Should the Principal Investigator's RO1 grant be renewed, her salary and effort would be covered to 85%. The remaining 15% is requested on this grant. The high cost of living in New York City necessitates competitive salaries.

*Fringe benefits:* Cornell University Medical College fringe benefits from 12/1/94 to 11/30/95 are at 32%. Salary increases of 4% are in accordance with Cornell University Medical College guidelines. Cornell University Medical College documentation of calculation of the indirect cost and fringe rate are attached.

*Equipment:* Our equipment situation is reasonably good, as most of our equipment was bought 8 years ago. We are continuously looking for ways to salvage equipment and cut costs. Due to continuing budget cuts, Dr. William Holloman (Professor at Cornell, shares laboratory space with the Principal Investigator) and the P.I. have made a concerted effort to pool our resources and share as much equipment as possible. This occasionally means re-arranging our experiments while waiting several days for the FPLC to be free, but it has worked out quite well and strengthened the interactions between our two laboratories. When I had the only thermal cycler, both labs co-ordinated its use without difficulty. We have shared in the expense of shared computers, computer programs such as DNA-Star, gel dryers, vacuum pumps, and service contracts. No equipment costs are requested for this grant for the first year. Replacement of used equipment and ability to buy smaller items is requested for years 2-5.

Our capacity to synthesize large numbers of oligonucleotides, perform large numbers of LCR, LDR, and PCR amplifications, and fluorescent quantification of LDR products on a DNA sequencer has been tremendously augmented by generous equipment gifts from Roche Molecular Diagnostics, Perkin Elmer, and Applied Biosystems Inc. Specifically, we now have our own PE 9600 thermal cycler, ABI 394 DNA synthesizer, and an ABI 373 automated DNA sequencer. Although these instruments were placed in my laboratory for use in developing the Ligase Chain Reaction (LCR) for detection of infectious and genetic diseases and isolating new thermophilic proteins, all three companies have given their encouragement to use these instruments for cancer research. Such equipment still necessitates highly skilled and motivated personnel to run them. See Core B.

*Supplies.* We are perpetually underfunded in these categories, but the submitted budget is in compliance with our past levels of support. Our DNA modifying enzymes include restriction enzymes (we make our own *TaqI*), T4 kinase, T4 Ligase, Klenow, *Taq* Polymerase, and other enzymes. Chemicals and reagents include all our chemicals, column matrixes, miniprep columns, as well as chemicals required for oligonucleotide synthesis. Other supplies include radioisotopes, media, and film.

*Travel:* We request support for the Principal Investigator or the Co-investigator to attend one domestic meeting per year in order to present results and learn of advances related to Breast Cancer Genetics and Cancer Detection discussed in this proposal.

*Other expenses.* We request support for phone, Fax, printing, and photocopying of \$3,000 for the first year. The results from an automated fluorescent LDR detection experiment are printed in color to distinguish marker from experimental peaks. This necessitates use of color printers or color copying machines.

**RESOURCES AND ENVIRONMENT**

FACILITIES: Mark the facilities to be used at each performance site listed in Item 9, Face Page, and briefly indicate their capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Use "Other" to describe the facilities at any other performance sites listed in Item 9 on the Face Page and at sites for field studies. Use continuation pages if necessary. Include an explanation of any consortium/contractual arrangements with other organizations.

☒ Laboratory: The Barany group currently numbers 6 full time researchers including the P.I., and one part time technician. The lab is on the fourth floor of the microbiology wing, and comprises 3,500 sq. ft. of relatively new space (8 yrs since renovation.) The Barany lab is 670 sq. ft., in addition, a cold room, an equipment room, a dark room and a computer room are shared with Dr. William Holloman.

☐ Clinical:

☐ Animal:

☒ Computer: The P.I. has a Macintosh Quadra 840 AV (in his office), four Macintosh IIfx, and one Macintosh Classic computer which is used primarily to program our HPLC. Our Microbiology Dept. has a SPARCstation 2 with the Genetics Computer Group package of programs for retrieval and analysis of protein and DNA sequences. We are also directly connected with the Rockefeller University computer.

☒ Office: The P.I. has a private office of about 180 sq. feet.

☐ Other ( ): \_\_\_\_\_

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

With Dr. Holloman, the P.I. share one ultracentrifuge, 2 high speed and 2 low speed centrifuges, several microfuges, a liquid scintillation counter, 2 Vis/UV spectrophotometers (one with kinetics), a fluorescence spectrophotometer, 2 PE thermal cyclers, an FPLC, an HPLC, 3 ultralow freezers, a fermenter, 2 floor shakers, 2 chemical hoods, a biological hood, a french press, a sonicator, a lyophilizer, a speed vac, a rotary evaporator, several water bath shakers, fraction collectors, gel dryers, power supplies, freezers, refrigerators, and incubators. Our department has a phosphorimager which is shared among our faculty. In addition, Roche, PE, and ABI, have placed a PE 9600 thermal cycler, an ABI 394 DNA synthesizer, and an ABI 373 automated DNA sequencer in my laboratory with encouragement to use these instruments for research on the Cancer detection studies, as well as for the other projects.

ADDITIONAL INFORMATION: Provide any other information describing the environment for the project. Identify support services such as consultant, secretarial, machine shop, and electronics shop, and the extent to which they will be available to the project.

Our department of Microbiology has grown to 7 members under the leadership of Dr. Kenneth I. Berns. He has forged a joint Graduate program in Molecular Biology with Dr. Jerry Hurwitz at our neighboring Sloan Kettering Memorial Research Institute, so our joint department numbers 30 faculty members. We have full access to the core facilities at Sloan Kettering. Dr. Neil Hackett is within our department, and Dr. Mathew Lubin is At the Strang Cancer Prevention center across the street. In addition, I have an adjunct appointment at The Rockefeller University (also across the street) in the Department of Chemistry, Biochemistry, and Structural Biology, now headed Dr. David Cowburn. Finally, my X-ray collaborator, Dr. Aneel Aggarwal is a few miles uptown at the College of Physicians & Surgeons, Columbia University. Thus, we can all easily get together to discuss new results and ideas.

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**RESOURCES AND ENVIRONMENT**

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**FACILITIES:** Mark the facilities to be used at each performance site listed in Item 9, Face Page, and briefly indicate their capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Use "Other" to describe the facilities at any other performance sites listed in Item 9 on the Face Page and at sites for field studies. Use continuation pages if necessary. Include an explanation of any consortium/contractual arrangements with other organizations.

☒ **Laboratory:** The Strang-Cornell laboratory space totalling about 4500 Square feet is located at 510 East 73rd Street, a short walk from the Strang Cancer Prevention Center's offices and the Cornell Medical Center. The facility includes a cold room and tissue culture room with laminar flow hood. Approximately 320 square feet are dedicated for use by Dr. Lubin.

☒ **Clinical:** The Strang Cancer Prevention Center has two main resources for clinical investigations. Patient visits at Strang come to more than 5000 per year. Of these approximately 4000 are for breast cancer screening, preoperative and follow up evaluations for breast cancer surgery, and breast cancer chemotherapy. The Strang National Registry for women with family histories of breast cancer maintains and updates family history information on over 14,000 women. All clinical investigations at Strang are approved by Strang's IRB. We also enjoy the close cooperation of the Department of Pathology and the Division of Oncology at the New York Hospital-Cornell Medical Center.

☐ **Animal:**

☒ **Computer:** Dr. Lubin's laboratory is equipped with a Macintosh SE/30 computer and StyleWriter printer. In the office he has a Macintosh Iici with a LaserWriter printer and a PowerBook 140.

☒ **Office:** Dr. Lubin's office is located at the Strang Cancer Prevention Center at 428 East 72nd Street. His office space amounts to approximately 150 square feet.

☐ **Other ( ):**

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**MAJOR EQUIPMENT:** List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.  
Equipment includes: Ultracentrifuge (Becman, L8-70m). UV-Visible spectrophotometer (Perkin-Elmer). Beta counter (Pacard Tricarb 300). Thermal Cycler (Perkin-Elmer 9600). Speed-Vac concentrator. Gamma counter (LKB). Freezers -20 and -70 degrees. Tissue culture facilities.

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**ADDITIONAL INFORMATION:** Provide any other information describing the environment for the project. Identify support services such as consultant, secretarial, machine shop, and electronics shop, and the extent to which they will be available to the project.

The Strang Cancer Prevention Center is across the street from Cornell University Medical College and the New York Hospital. This close proximity facilitates the collaboration between the P.I. and Co-I.

## A. SPECIFIC AIMS:

This proposal represents a new approach to multiplex detection of point mutations, gene amplifications and deletions in tissue samples associated with breast and cervical cancer. We plan to combine polymerase chain reaction (PCR) with ligase detection reaction (LDR, developed in the P.I.'s laboratory) to provide highly sensitive and specific identification of genetic lesions in breast tumors. We plan to achieve the following three objectives and apply them to primary tumor, lymph node, and bone marrow specimens collected from 100 to 200 well-characterized breast cancer cases.

(i) **Development of a multiplex polymerase chain reaction/ligase detection reaction (PCR/LDR) system for detection of point mutations in tumor biopsies.** Our laboratory has developed a PCR/LDR method for multiplex discrimination of ten gene conversion mutations which cause 21 hydroxylase deficiency. This PCR/LDR technology will be extended to identify cancer causing mutations in the p53 tumor suppressor gene in a variety of characterized cell lines and "touch prep" DNA samples. By optimizing ligation conditions and/or using mutant *Tth* ligase, we aim to increase the sensitivity of this assay to detect 1 cancer gene mutation in  $10^2$  to  $10^3$  normal cells (See Project 4). We plan to develop this assay to simultaneously detect possible mutations in any one of 24 to 40 codons in the p53 gene in frozen and fixed primary breast tumors from the 100 to 200 breast cancer cases. The PCR/LDR technology will also be used to type HPV strains in cervical biopsy specimens which correlate with cervical cancers. Oligonucleotide or PNA addressable arrays will allow for simultaneous screening of dozens to hundreds of potential mutations (See Project 5 and Core 2).

(ii) **Development of a multiplex ligase detection reaction/polymerase chain reaction (LDR/PCR) system for detection of gene amplifications and deletions in tumor biopsies.** This study aims to develop a technology to investigate the amplification and deletion of several genes in a single assay. We have designed ligase detection primers for the following genes: SOD (on chromosome 21q), G6PD (on chromosome Xq), p53 tumor suppressor gene (on chromosome 17p), HER-2/neu (on chromosome 17q), and Int-2 (on chromosome 11q). Each set of LDR primers includes the same external "zip code" sequences, which will allow for proportional amplification of all five chromosome probes with a single pair of PCR primers. The method will be validated with genomic DNA samples from normal, and trisomy 21 human males and females. Subsequently, cell lines with known HER-2/neu and int-2 gene amplifications, and p53 deletions will be used to test the sensitivity of this technique. The assay will be extended to frozen and fixed breast tumor material. Initial studies will investigate the 100 to 200 tumors to determine the feasibility of large scale studies correlating multiple genetic alterations to the clinical/biological behavior of breast cancers.

(iii) **Development of a polymerase chain reaction/restriction endonuclease/ligase detection reaction (PCR/RE/LDR) to detect and identify mutations at a sensitivity of 1 in  $10^6$  or  $10^7$  cells.** A general method to detect any rare cancer cell carrying a mutation in any gene has been developed. The principle of this method is to continuously remove normal sequences while selectively amplifying the cancer mutation. A two base region of interest is amplified using PCR primers creating a restriction endonuclease recognition site. Treatment with the cognate endonuclease selectively cleaves product arising from the wild-type sequence. The cancer mutation resists cleavage, and thus is selectively amplified. Use of LDR or LCR primers distinguishes the authentic mutation from background primer dimer or polymerase misincorporation products during the detection step. This method will be expanded to demonstrate conversion of *any* dinucleotide (16 possibilities) into one of nine special endonuclease recognition sites. Use of multi-pairing or universal spacer nucleotide analogues will enhance significantly the efficiency and specificity of several of these site conversions (See Project 3). The p53 gene codon 248 has been converted to a *TaqI* site, demonstrating the feasibility of this approach. We plan to extend the technology to include conversion of breast cancer "hot spot" codons V157, R175, R273, and R282 into *TaqI* sites. Should primary breast tumors contain mutations in these 5 hot spots, lymph nodes and bone marrow samples from the same patients will be tested by PCR/RE/LDR for the presence of tumor cells. In this initial study, the presence of micrometastases, will be correlated with HER-2/neu and int-2 gene amplification.

## BACKGROUND AND SIGNIFICANCE

There has been a deeper understanding of carcinogenesis due to recent advances in molecular biology. Cells at various stages tumor development demonstrate deletions, insertions and point mutations in oncogenes and tumor suppressor genes. The ras oncogene, the p53 gene and the adenomatous polyposis coli (APC) gene have been implicated in the development of tumors of the brain, breast, bladder, stomach, lung, liver, and colon [1-14]. Early detection of mutations, such as identifying K-ras mutations from shed colorectal tumor cells, has already demonstrated clinical utility [15].

One of the goals of cancer genetics research is to predict the biological and clinical behavior of tumors from their genetic abnormalities. N-myc gene amplification correlates well with staging in neuroblastomas [16]. A primary neuroblastoma with N-myc amplification or deletion of chromosome 1p has a poor prognosis and is rapidly fatal in general, even if discovered at an earlier stage. Near-diploid tumors without N-myc amplification or 1p deletions show a slow progression and are often fatal. Hyper-diploid or near triploid neuroblastomas have generally have good outcomes (reviewed in [17].) Therefore, genetic analysis can influence prognosis and direct treatment choices in cases of neuroblastoma.

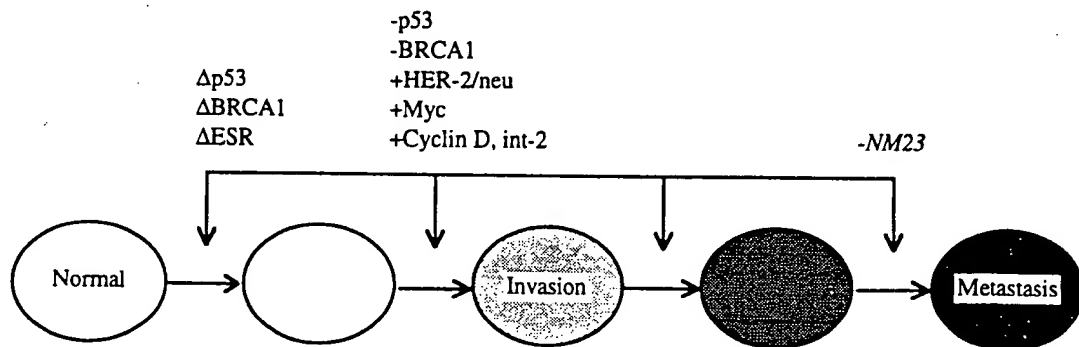


Fig. 1. The Mary Claire-King multi-step model for the development of breast cancers. The symbol  $\Delta$  indicates a locus which may contain a germline mutation. (-) = gene deletion, (+) = gene amplification. Adapted from [18].

The genetic alterations in adult-onset cancers are considered to be more complex than in pediatric tumors. A multi-step model for the development of colon cancers is generally accepted [9] and a similar model for breast cancer carcinogenesis has been proposed [18]. (See Fig. 1, above.) Currently the best indicator of prognosis in breast cancer is the number of disease-affected lymph nodes at the time of diagnosis [19]. The prognostic utility of oncogenes, their mRNA and protein products, tumor suppressor gene mutations and other tumor markers has been explored in node negative breast cancers. In 1990 an NIH Consensus Conference identified, as an area for future research, the development of risk factor profiles, "to allow identification of such groups that (a) may be treated with surgical excision with irradiation, (b) do not require axillary node dissection, and do not require systemic therapy" [20]. Many investigators have studied DNA, RNA and proteins as disease markers for their prognostic significance before and since that conference (reviewed in [14]), but their clinical utility has yet to be proven.

The best clinical indicator of breast cancer prognosis is the number of disease-affected lymph nodes at the time of diagnosis [19]. The utility of tumor suppressor gene mutations and oncogene expression on the mRNA and protein levels have been investigated in breast cancers as predictors of outcome, especially for their significance in "node-negative" cases. (Reviewed in [14]). Overexpression of p53 measured by immunohistochemical staining correlates with earlier relapse and shortened survival [21-23]. Mutations in the p53 gene in breast cancers are similarly associated with poor outcome [24-26]. However, DNA analysis may be more informative than immunohistochemical analysis, since different mutations have different effects on the p53 protein and its function [27-29]. For example, p53 proteins with mutations in exons 5 and 6 are capable of binding heat shock protein (hsp) 70 and are immunogenic. Mutations in exons 7 and 8 do not allow p53 to bind hsp 70.

Studies of amplification and overexpression of oncogenes in breast cancers have focused on the HER2/neu, c-myc and int-2 genes. HER-2/neu gene amplification correlates with mRNA and protein overexpression [30]. HER-2/neu amplification and overexpression may be useful indicators of prognosis in breast cancer [31-34]. Overexpression of this gene and the *ras* gene product may act in synergy, producing a poor clinical outcome [35]. Similarly, amplification in the c-myc gene has been correlated with poor prognosis in breast cancer [36, 37] but other studies have not found the same result [38, 39]. Amplification of the int-2 gene has also been associated with large tumor size, reduced time to relapse and shorter survival in breast cancer cases [38].

While these studies often show statistical significance between breast cancer prognosis and the gene amplification or its expression, there are conflicting results and their clinical utility does not surpass other prognostic factors. It is very likely that the full nature of breast cancer carcinogenesis is extremely complex. To understand the genetic progression and different subsets of breast cancer, clinical and basic scientists will have to continue to make correlations between the biological characteristics of tumors and their clinical outcomes. To succeed in this, we will have to be able to investigate more genes from smaller amounts of tumor tissue. DNA technology holds great promise for unlocking the vast amount of information stored in breast cancers, because it can amplify information without alteration.

Often, viral and cellular gene products interact to cause cancers. Molecular and epidemiological studies implicate Genital human papillomaviruses (HPV's) as contributing factors in cervical, vaginal and vulvar intraepithelial neoplasia and carcinoma [40, 41]. Infection with high risk HPV types 16, 18, 31, 33, 45, 51, correlates with an increased risk of carcinoma development, and recent studies have tried to associate the grade of cervical intraepithelial neoplasia to HPV type [42-44].

The transforming activity of the high risk groups is thought to be brought about by active transcription of the E6 and E7 open reading frames, implicating the protein encoded by these two genes to be important in the malignant phenotype [45, 46]. These viral proteins are thought to exert their effect by interacting with normal cellular proteins associated with the regulation of cell growth. The E7 proteins associated with high risk HPV types 16 and 18 form tight complexes with the retinoblastoma tumor suppresser gene (p105-RB) while HPV's from low risk groups (HPV- 6b and HPV-11) bind to p105-RB with a lower affinity [47, 48]. Similarly, the E6 protein from HPV's 16 and 18 binds to, and stimulates the degradation of the tumor suppresser protein p53 [49]. Thus expression of the viral E6 and E7 proteins from the high risk HPV types corrupts normal control mechanisms that regulate cell proliferation by binding to p53 and p105-RB, stimulating infected cells to grow and produce new virus particles. In doing so, they provide the potential for malignant growth [50, 51].

Risk assessment due to HPV infection is conceptually simple; one merely has to look for sequences derived from the high risk HPV types in cervical lavages or biopsies. Patients with pathologically abnormal tissue will be treated regardless of virus type, thereby elevating the necessity to screen for HPV types outside the high risk group. Typing is of particular importance in patients with no obvious lesion or low grade lesions but who are infected with a high risk strain. Thus, there is an immediate clinical need for a rapid and accurate method for simultaneously detecting even low level infections with high risk HPV strains.

There is a three-fold challenge to detection of cancer-associated gene alterations. First, a myriad of cancer causing mutations must be detected. A recent review of p53 gene mutations in cancers identified mostly point mutations distributed over 108 codons [52]. Second, oncogene amplifications and tumor suppressor gene deletions must be accurately quantified. Once the mutations in a primary tumor are identified, a third challenge would be to detect the spread of cancer cells to lymph nodes, bone marrow or blood. This would require the sensitivity to detect a mutation present in only one cell out of  $10^6$ . The complementary specificity's of *Taq* polymerase and *Tth* ligase will be exploited to combine PCR (polymerase chain reaction) with LDR/LCR (ligase detection reaction /ligase chain reaction) to achieve exquisite specificity's [53-55]. This background section will briefly discuss current approaches and our recent progress towards solving the three-fold challenge to cancer detection as outlined below:

(a) Detection of single base mutations.

- (i) *Methods based on the polymerase chain reaction (PCR).*
- (ii) *The ligase chain reaction (LCR) for detection of single base mutations.*
- (iii) *Multiplexed detection of point mutations with the PCR/LCR and PCR/LDR.*

(b) Detection of gene amplifications and deletions.

- (i) *PCR based methods for detecting gene amplifications and inherent problems.*
- (ii) *A new approach for gene quantification using LDR/PCR.*

(c) Detection of mutations at 1 out of  $10^6$  or even  $10^7$  cells.

- (i) *PCR based methods for detecting mutations at high sensitivity.*
- (ii) *A generalized method for detecting any mutation at high sensitivity.*
- (iii) *Development of assays for detecting rare mutations at CG dinucleotides by conversion to TaqI recognition sites.*

We wish to point out the rapid advances in discovering new mutations associated with disease causing genes or cancers [56]. An elegant method which amplifies and clones large and small differences between two genomes shows great promise for identifying infecting viruses, genome translocations/ rearrangements, or point mutations which have been associated with tumor development [57, 58]. Once such macroscopic alterations within the genome are identified, PCR and LDR technology may be combined to rapidly screen for such rearrangements in other tissue from the same patient or other patients. The DNA detection methods may thus help determine the significance of gene rearrangements or mutations isolated by difference cloning.

The presence of small deletions, insertions, or single base differences between a normal and mutant gene may be rapidly detected by use of SSCP and rSSCP [59-61], RNase or chemical cleavage of mismatched heteroduplexes [62-64], or even direct sequencing [13, 65, 66]. Clever variations of sequencing, such as dideoxy fingerprinting (ddF) already demonstrated the ability to detect presence of 84 out of 84 (=100%) different mutations with a very low rate of false positive signals [67]. As an alternative to sequencing, functional screens for germ line mutations based on DNA binding activity have been elegantly developed for p53 [68, 69]. These screens are quite valuable since they distinguish cancer causing mutations from normal human polymorphisms.

(a) Detection of single base mutations:

(i) *Methods based on the polymerase chain reaction (PCR).* Single base mutations may be detected using allelic specific PCR, nested PCR, PASA or double ARMS amplification [65, 70-75]. Yet diagnostic use in the clinic has met certain limitations. PCR has difficulty distinguishing single base deletions or insertions in a poly N tract. PCR requires careful optimization to detect a cancer mutation in the presence of normal tissue. Furthermore, PCR is not optimal for simultaneous detection of several mutations in a single reaction (known as multiplexing). When several mutations are on a single gene, such as the p53 tumor suppressor gene, multiple PCR primers may run into each other during amplification. These problems can be overcome by combining LCR with PCR.

(ii) *The ligase chain reaction (LCR) for detection of single base mutations.* The P.I. has developed a powerful method for discriminating single base mutations, termed ligase chain reaction or LCR. *Tth* ligase was employed in an assay that both amplifies DNA and discriminates a single base substitution [54, 55]. This cloned enzyme specifically links two adjacent oligonucleotides when annealed at 65°C to a complementary target only when the nucleotides are perfectly base paired at the junction. Oligonucleotide products are exponentially amplified by thermal cycling of the ligation reaction in the presence of a second set of adjacent oligonucleotides, complementary to the first set and the target. A single base mismatch prevents ligation/amplification and is thus distinguished. (See Fig. 2) Use of four primers gives an exponential amplification and is termed LCR. Use of only two adjacent primers gives a linear amplification, and is termed ligase detection reaction or LDR. LCR and LDR have the advantage of being compatible with PCR, allowing for multiplex detection of single base mutations.



**LCR**

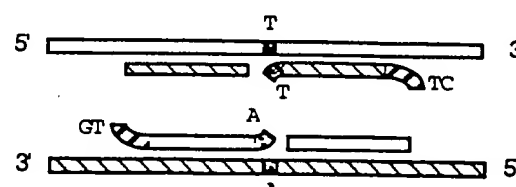
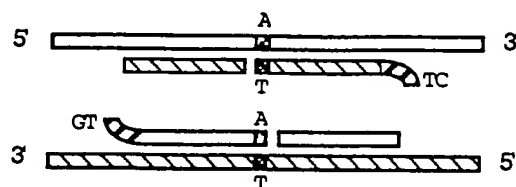
## Complementary Target:

 $\beta^A$  Globin

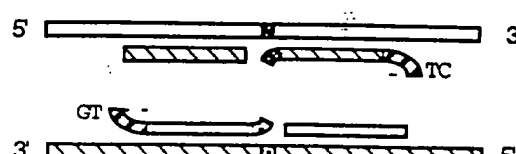
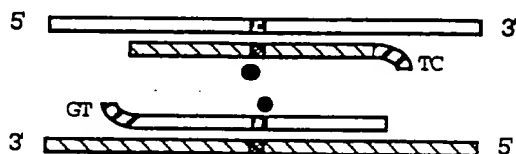
## Mismatched Target:

 $\beta^S$  Globin

1. Denature DNA, 94°C. Anneal oligonucleotides, 65°C.



2. Ligate with thermostable ligase at 65°C.



3. Repeat cycle 20 to 30 times.

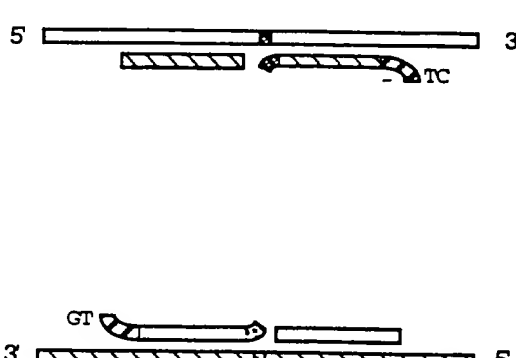
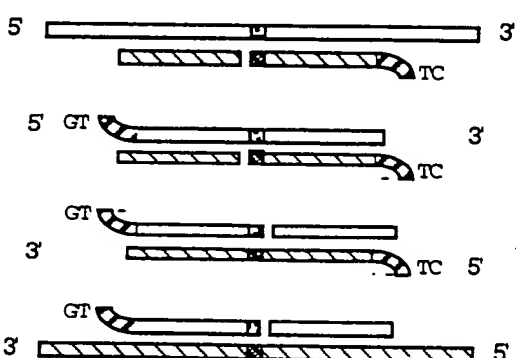


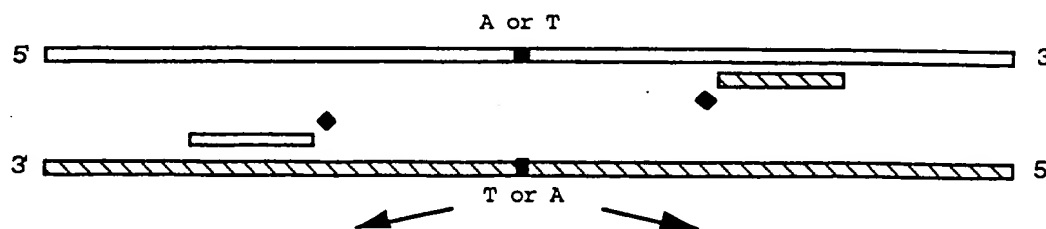
Fig. 2. Allele-specific DNA amplification and detection using the ligase chain reaction (LCR). DNA is denatured at 94°C and the four LCR primers anneal to their complementary strands at 65°C, near their melting temperatures ( $T_m$ ). Thermostable ligase (depicted as a black circle) will only ligate adjacent primers that are perfectly complementary to their target sequences. On the left a complementary target ( $\beta^A$ ) allows ligation. A single base-pair mismatch at the internal 3' end of the adjacent primers prevents ligation (as shown in the case of  $\beta^S$  on the right). A pair of primers complementary to  $\beta^S$  could be used to detect this mutation. The discriminating primers have non-complementary GT or CT tails at their 5' ends to avoid ligation in the wrong orientation.

(iii) *Multiplexed detection of point mutations with the PCR/LCR and PCR/LDR.* The ligase chain reaction is ideal for multiplexing. Since there is no polymerization step, several primer sets can ligate along the length of a gene without interference. The optimal multiplex detection scheme involves a primary PCR amplification, followed by either an LCR (four primers, both strands) or LDR (two primers, same strand) detection (See Fig. 3). This approach has been successfully applied to multiplex detection of cystic fibrosis [76-78], hyperkalemic periodic paralysis [79], and 21 hydroxylase deficiency (D. Day, P. White, and F. Barany, unpublished). In the 21 hydroxylase deficiency project, we have developed methodologies which rapidly determine heterozygous or homozygous individuals for any of the ten common gene conversions in *CYP21* (See Preliminary Results). The method was able to distinguish insertion of a single T nucleotide into a (T)<sub>7</sub> tract, which can not be achieved by PCR alone. Likewise, PCR/LDR multiplexing was used to distinguish 30 cystic fibrosis mutations, even when such changes were separated by only a single codon [78]. In this work we plan to use PCR/LDR multiplexing to simultaneously detect dozens of mutations in the p53 tumor suppressor gene.

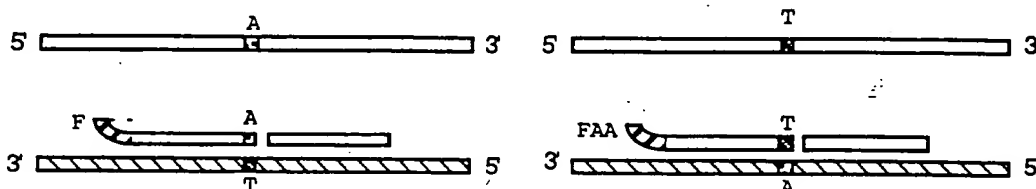


**PCR/ LDR****CYP21 exon 4**

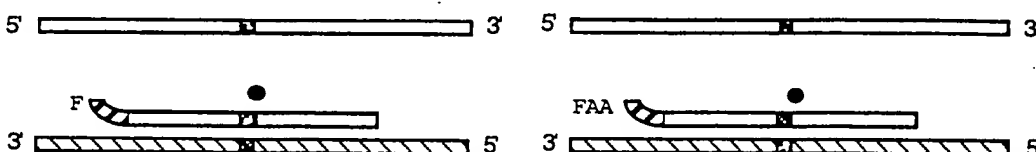
1. PCR amplify using common oligo-nucleotides, dNTPs and *Taq* polymerase. Dilute into second tube.



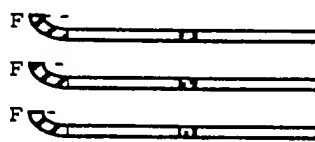
2. Denature DNA, 94°C. Anneal common and fluorescently labeled, allele specific oligonucleotides, 65°C.



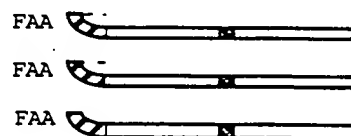
3. Ligate with thermostable ligase at 65°C.



4. Repeat cycle 2 to 10 times. Separate fluorescent products on DNA sequencer and quantify each allele.



CYP21b, I172,  
Wild type gene



CYP21a, N172  
Pseudogene mutation

Fig. 3. Allele-specific detection and quantification using the polymerase chain reaction with the ligase detection reaction (PCR/LDR). The fragment of interest is amplified by a primary PCR reaction using *Taq* polymerase (depicted as a black diamond). DNA is denatured and two LDR primers anneal to their complementary strands. The allele specific LDR primers have discriminating bases on their 3' ends and different length "tails" at their 5' ends. The allele-specific LDR primers will only ligate to the adjacent common primer when there is no mismatch at the junction. If the common primer is limiting, the allele specific products generated are proportional to the alleles present in the starting target DNA. In this example PCR is used to amplify exon 4 in the CYP21 (steroid 21 hydroxylase) gene. Allele-specific primers contain fluorescent groups at their 5' ends on different length ploy-A tails. The discriminating bases are at their 3' ends. Equimolar ligation of both allele specific primers indicates the individual is heterozygous. PCR/LDR may be used in a multiplex format, in which 30 primers can distinguish 20 individual alleles in the same reaction.

**(b) Detection of gene amplifications and deletions.** Gene alterations in tumor cells include point mutations, as well as gene deletions and amplifications. Amplification of the N-myc gene has been shown to have important prognostic significance in neuroblastoma [16, 17]. The prognostic significance of gene amplifications (e.g. HER-2/neu, c-myc and int-2) in breast cancers has not been clearly established, perhaps because adult-onset tumors are more complex than pediatric cancers.

**(i) PCR based methods for detecting gene amplifications, and inherent problems.** Accurate quantification of nucleic acids using PCR has been achieved by co-amplifying artificial template DNA's as references to the native gene sequences [80]. The artificial templates are constructed containing the same primer sequences but different internal sequences. Because they have identical primer sequences, the native gene and the artificial template appear to have identical amplification efficiencies. Thus the ratio of PCR products reflects the ratio of the native gene and the template at the start of the reaction. Attempts to quantify two or more genomic DNA sequences simultaneously using one as an endogenous, internal control for the

others have been more problematic. Different primer pairs have different PCR efficiencies. After several cycles of amplification, the PCR products are no longer in the same proportion as at the start of the reaction. Controlling for this effect requires careful design of PCR primers and experimental conditions [81].

(ii) *A new approach for gene quantification using LDR/PCR.* The ligase detection reaction may prove useful for demonstrating gene deletions and amplifications. The general concept is to use sets of gene specific LDR primers to generate artificial PCR templates with unique internal sequences and identical external sequences which can be used for PCR amplification. After an initial LDR step, the ratio of the products should reflect the ratio of starting templates. Since all LDR products have the same outside sequences, they can be amplified by a common pair of PCR primers, which we term "zip code" primers. Since the primer sequences, G + C content and fragment lengths are designed to be identical, the efficiencies of amplification should be nearly equal. Thus, the ratio of PCR products should reflect the ratio of genes at the start of the reaction. By combining LDR with PCR, we plan to accurately quantify gene deletions in p53 and gene amplifications in HER-2/neu and int-2, as described in Methods section (ii).

(c) Detection of mutations at 1 out of  $10^6$  or  $10^7$  cells.

(i) *PCR based methods for detecting mutations at high sensitivity.* An extremely accurate method for identifying K-ras mutations from shed colorectal tumor cells in feces combines PCR, phage cloning, and radioactive probe detection [15]. The approach could accurately identify mutations at the 1% level and lower, but is technically challenging and requires a biological phage plating step. An alternative approach requires exceedingly careful optimization of both primers and conditions for allelic specific PCR amplification of K-ras mutations [82]. The method could detect a few cells containing mutant K-ras gene present in the blood of patients with pancreatic adenocarcinoma. This PCR method shows significantly less sensitivity for distinguishing transition mutations compared to transversions. Nevertheless, the above methods demonstrate the clinical utility of being able to identify rare mutations in cancer causing genes.

(ii) *A generalized method for detecting any mutation at high sensitivity.* Our generalized cancer detection method is based on removal of the normal sequence while selectively amplifying the cancer mutation. When fully developed, this method should be able to quantify the level of a known mutation anywhere from 1 in  $10^2$  to 1 in  $10^7$  normal cells. An earlier and far less versatile form of this concept is illustrated in a method which detects the presence of cancer causing mutations at a TaqI site in the human c-H-ras1 gene [83]. Presence of a mutation (T $\overline{C}$ GA to T $\overline{T}$ GA) destroys a pre-existing TaqI site, and the cancer mutation may be selectively amplified by adding TaqI after cycles 1, 3, 5, 7, etc. of PCR. This method can rescue and detect 10 mutated copies in  $10^8$  wild-type sequences; however, it requires laborious cloning and sequencing for quantification. In collaboration with Dr. Vincent Wilson, we have developed a simple PCR amplification /endonuclease selection /LCR detection method (PCR/RE/LCR) to achieve even greater sensitivity (See Project 1). Preliminary studies in the Ha-ras gene have demonstrated detection of 10 mutated copies in  $10^9$  wild-type sequences [84-86]. The reported fidelity of Taq polymerase suggested that misincorporation of bases would limit this PCR/LCR procedures' sensitivity to less than one in  $10^6$  [87, 88]. However, the fidelity of Taq polymerase was determined as a misincorporation in any base within a large amplified region and the fidelity actually increases for any specific individual base. These PCR/LCR procedures have not falsely detected a mutation in more than 12 separate analyses of the wild type DNA. (Similar approaches of PCR/endonuclease detection of Ha-ras gene mutations give less sensitivity since they lack the LCR detection step [89-95]).

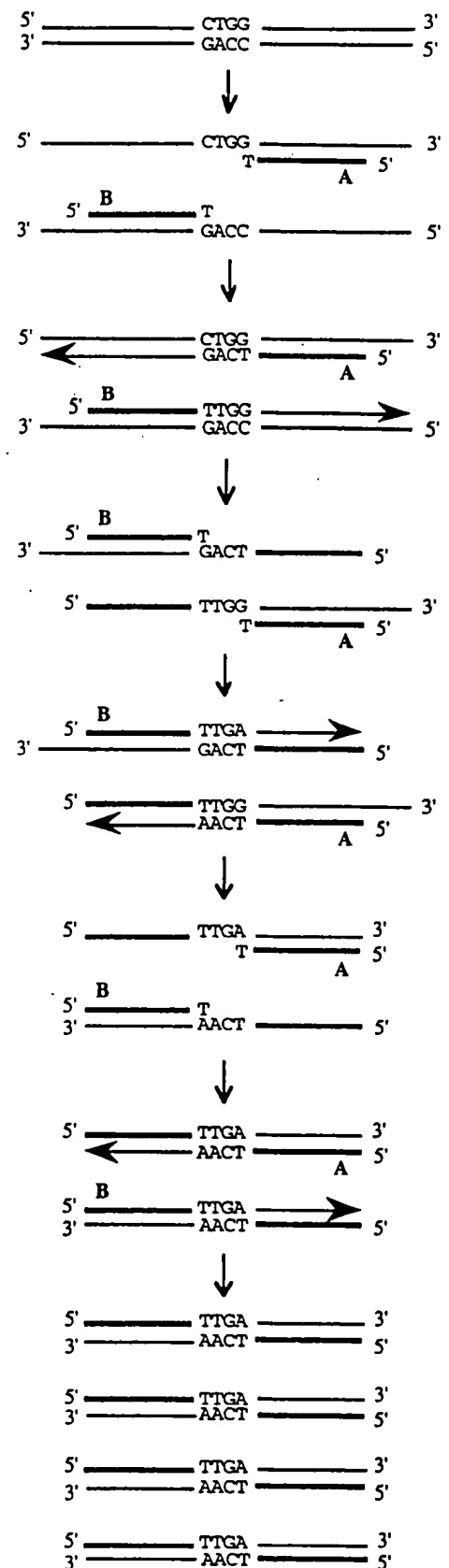
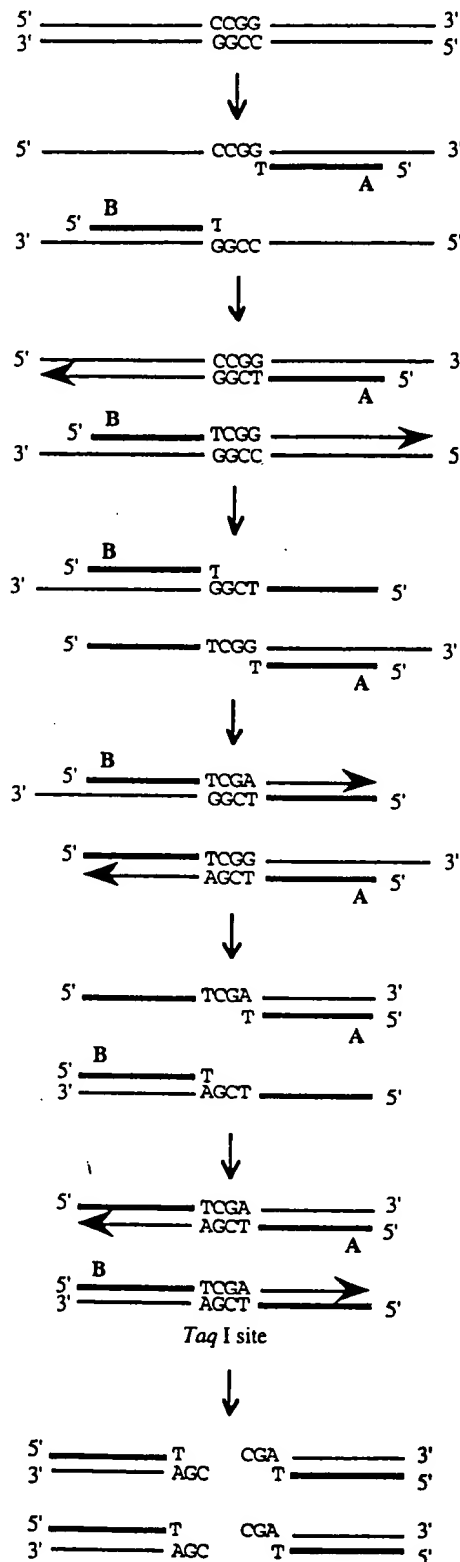
(iii) *Development of assays for detecting rare mutations at CG dinucleotides by conversion to TaqI recognition sites.* We now have developed a generalized method for converting every pre-existing CG dinucleotide sequences in a cancer-causing gene into a PCR product containing a TaqI restriction endonuclease site (T $\downarrow$ CGA) at that position (See Fig. 4). Mutations at CG dinucleotides account for a large percentage of all mutations in human genes (presumably due to spontaneous or enzymatic deamination of 5-methyldeoxycytidine [96-101]). PCR is used to amplify a two base region in both the majority normal and minority cancer DNA. By judicious design of the primers, a new restriction site is created within the normal--but not the mutated DNA. The two PCR primers are perfectly complementary to the DNA flanking the CG dinucleotide, except for a 3' T nucleotide, independent of whether it matches the actual nucleotide flanking the CG dinucleotide (See Fig 4, step 2). By using very rapid PCR amplifications at high dNTP concentrations, this mismatched T is extended [71, 90]. After the first round of amplification each primer generates a sequence of

PCR/ RE/ LDR

### Normal Sequence

### Cancer Sequence

1. Denature DNA at 94°C.
2. Anneal primers A and B.  
Both primers contain a T at their 3' end.
3. Extend with *Taq* Polymerase at a dNTP's concentration of 800 uM.
4. Denature DNA at 94°C.  
(Only newly synthesized DNA shown - for clarity.)
5. Anneal primers A and B.
6. Extend with *Taq* Polymerase.
7. Denature DNA at 94°C.  
(Only newly synthesized DNA shown - for clarity.)
8. Anneal primers A and B.
9. Extend with *Taq* Polymerase.
10. Normal DNA is cut by *Taq* I endonuclease. Cancer DNA is not cut, and continues to amplify.



**Fig. 4. A generalized method to detect and identify mutations at a sensitivity of 1 in  $10^6$  or  $10^7$  cells. See text.**

the form TCGN (Step 3). Since the second round of amplification uses the complementary primer, this sequence is converted into TCGA, the *TaqI* site that was sought (Step 5). A cancer mutation (TG) would be converted to TTGA sequence, which is refractory to *TaqI* cleavage. During a second set of PCR amplifications, normal DNA is cleaved by three reiterative additions of a thermophilic restriction endonuclease, in this example *TaqI* (Step 10). A final LDR step detects the actual cancer causing mutation [54]. The ligase detection step allows for accurate quantification of the amount of original cancer mutation, as well as avoiding false positive signals from primer dimers. This concept has been generalized to include 9 restriction endonucleases, which can detect all 16 possible dinucleotide pairs, and hence any cancer mutation at 1 in 10<sup>6</sup> sensitivity.

## C. PRELIMINARY RESULTS

### (i) Ligase chain reaction and ligase detection studies:

(a) *Genetic disease detection and DNA amplification using cloned thermostable ligase.* Thermostable DNA ligase was cloned and harnessed in an assay that both amplifies DNA and discriminates a single base substitution. This cloned enzyme specifically links two adjacent oligonucleotides when annealed at 65°C to a complementary target, only if the nucleotides are perfectly base paired at the junction. Oligonucleotide products are exponentially amplified by thermal cycling of the ligation reaction in the presence of a second set of adjacent oligonucleotides, complementary to the first set and the target. A single base mismatch prevents ligation/amplification and is thus distinguished. This method was exploited to detect 200 target molecules as well as to discriminate between normal  $\beta^A$  and sickle  $\beta^S$  globin genotypes from 10  $\mu$ l blood samples.

The P.I.'s laboratory, in collaboration with others, has used PCR coupled LDR/LCR detection to easily discriminate single base mutations in Leber's Hereditary Optic Neuropathy, *Listeria monocytogenes*, hematopoietic tumors, and Hyperkalemic Periodic Paralysis [54, 55, 79, 102-106].)

(b) *Detection of 21-Hydroxylase gene conversions associated with Congenital Adrenal Hyperplasia.* 21-hydroxylase deficiency is one of the commonest genetic diseases in man, and has the highest occurrence of any genetic disease with 6% of Ashkenazi Jews being carriers. The locus for 21-hydroxylase has two neighboring genes on the short arm of chromosome 6, the first encodes an inactive pseudogene named *CYP21P*, (*CYP21A*) while the other gene encodes an active gene, *CYP21*, (*CYP21B*, See Fig. 5) [107, 108]. The majority of mutations for 21-hydroxylase deficiency described to date are the result of recombination or gene conversion between *CYP21* and *CYP21P*. Mutations involving deletions or frame shifts cause the severe salt wasting form of diseases [109, 110], as do mutations Q318X, I235N, V236E, M238K and R356W, while many of the other mutations result in a reduction in 21-hydroxylase activity and are found in patients with non classic disease [111-113].

We have been developing methodologies that will enable the rapid determination as to whether a patient is heterozygous or homozygous for any of the 10 common apparent gene conversions in *CYP21*. By using allele specific PCR we have been able to amplify defined regions of *CYP21* (See Fig. 5). The presence of wild type or pseudogene mutation is subsequently determined using a fluorescent ligase detection reaction.

Allele specific amplification of *CYP21* has been obtained by designing PCR primers that are perfectly complementary to *CYP21* but contain mismatches at the 3' end of the primer to the *CYP21P* sequence. The *CYP21* and *CYP21P* sequences are sufficiently similar that the b-specific primers (Fig. 5) are able to anneal to the *CYP21P* sequence at the annealing temperature used for thermal cycling, but extension from the mismatched 3' end of the primer is prevented by using high fidelity PCR conditions and short extension times [65, 71]. For developmental purposes we have been amplifying the *CYP21* gene in short overlapping fragments to allow multiple independent assignments at each gene conversion site.

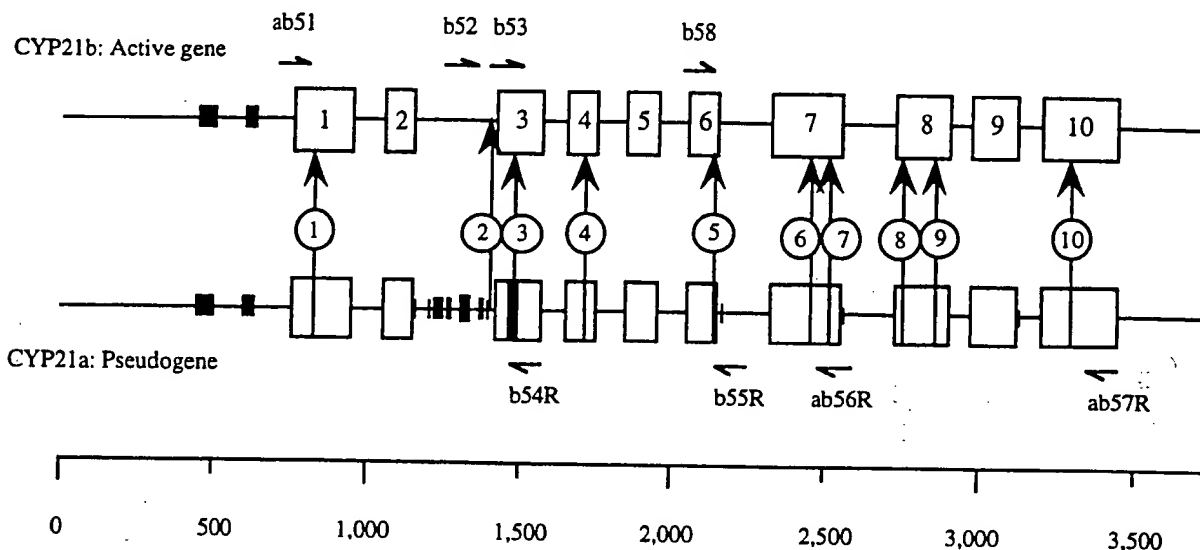


Fig. 5. Schematic of the structure and organization of *CYP21* mutations. Panel A; the *CYP* active and pseudogene which are located on the short arm of chromosome 6, are shown in relation to the two genes encoding the fourth component of serum complement C4A and C4B. Panel B; Sequence differences between the *CYP21P* pseudogene and *CYP21* active gene, known to cause clinical 21-hydroxylase deficiency are shown by the circled numbers: 1, exon 1, Pro-30→Leu; 2, intron 2 A→G; 3, exon 3, 8-bp deletion; 4, exon 4, Ile-172→Asn; 5, exon 6, cluster of mutations (Ile-236→Asn; Val-237→Glu; Met-239→Lys); 6, exon 7, Val-281→Leu; 7, exon 7, Phe 306 + T; 8, exon 8, Gln-318→term; 9, exon 8, Arg-356→Trp. Other vertical lines represent differences between *CYP21P* and *CYP21* that are apparently not functionally significant. The *CYP21* active was specifically amplified in various segments using combinations of the PCR primers shown above and below the *CYP* genes, relative to the sequence to which they hybridize. The "b" primers specifically hybridize to only the *CYP21* gene while the "ab" primers hybridize to both active and pseudogene. Adapted from [107, 112]. We have synthesized over 50 primers for allelic specific PCR amplification /LDR detection.

The LDR primers are designed such that the upstream oligonucleotide is fluorescently labeled and complementary to the bottom strand of either *CYP21* or *CYP21P* (See Fig. 6.) Discrimination of pseudogene and active gene in the LDR is determined by whether the 3' end of the fluorescent oligonucleotide forms a perfect match to the target that was PCR amplified. Ligation to a common unlabeled primer (downstream LDR oligonucleotide) can only occur if there is perfect match at the 3' end of the upstream fluorescent oligonucleotide [54, 114]. Thermal cycling in which product is denatured and the detecting oligonucleotides re-annealed for ligation, generates a linear increase in the amount of ligation product formed. Discriminating oligonucleotides complementary to both *CYP21* and *CYP21P* are included in equimolar amounts in a single reaction tube so that a signal for either active gene, pseudogene, or both is always obtained.

We have synthesized different length fluorescent primers for the pseudogene and active gene, such that each ligation product has a unique length. In each case the product for *CYP21P* is two base pairs longer than that for *CYP21* and the products from each downstream mutation site are four bp longer than the previous. The different length LDR products formed, are differentiated and quantified by electrophoresis and fluorescence detection using an Applied Biosystems 373A 4-color DNA sequencer with Genescan 672 software. By having unique sizes for each product we have been able to multiplex the detection assay to look at multiple gene conversions in one reaction tube. Fig. 7A shows typical scans for a wild-type individual and 7B shows some examples of from 21-hydroxylase deficient patients. The same genotypes were obtained for over 20 individuals tested using PCR/LDR as that obtained by Southern analysis [115]. Currently, we have been increasing the versatility of the detection system to allow a greater number of patients to be screened on a single denaturing gel by analyzing for all 10 possible gene conversions in one lane.

Mutation			LDR primer set	LDR product size bp	
				CYP21P	CYP21
P30L	22 mer 20 mer		Fam AA_____T Fam _____C	21 mer	43 41
Intron 2 splice mutation	25 mer 23 mer		Fam AA(A) <sub>n</sub> _____G_____G Fam(A) <sub>n</sub> _____C_____ (A) (C)	22 mer	47 45
8 base deletion exon 3	29 mer 27 mer		Fam AA(A) <sub>n</sub> _____T Fam (A) <sub>n</sub> _____GA GAC TA C	22 mer	51 49
I172N	30 mer 28 mer		Fam AA(A) <sub>n</sub> _____A Fam (A) <sub>n</sub> _____T	25 mer	55 53
Exon 6 cluster	31 mer 29 mer		Fam AA(A) <sub>n</sub> _____C A A A Fam (A) <sub>n</sub> _____T T T T	28 mer (A) <sub>N</sub>	59 57
V281L	28 mer 26 mer		Fam AA(A) <sub>n</sub> _____T Fam (A) <sub>n</sub> _____G	35 mer (A) <sub>N</sub>	63 61
T insertion	28 mer 26 mer		Fam AA(A) <sub>n</sub> _____T T T T Fam (A) <sub>n</sub> _____T T T	TTT T 39 mer (A) <sub>N</sub>	67 65
Q318* (term)	29 mer 27 mer		Fam AA(A) <sub>n</sub> _____T Fam (A) <sub>n</sub> _____C	42 mer (A) <sub>N</sub>	71 69
R356W	27 mer 25 mer		Fam AA(A) <sub>n</sub> _____T Fam (A) <sub>n</sub> _____C	48 mer (A) <sub>N</sub>	75 73
P453S	28 mer 26 mer		Fam AA(A) <sub>n</sub> _____T Fam (A) <sub>n</sub> _____C	51 mer (A) <sub>N</sub>	79 77

Fig. 6. Detection of 21-hydroxylase alleles using the ligation detection reaction (LDR). Each LDR contains 3 oligonucleotides. The two upstream detecting oligonucleotides are fluorescently labeled with Fam and differ in length by two bases due to a poly-A tail. The longer oligonucleotide is complementary to the CYP21P allele while the two base shorter oligo is complementary to CYP21. Either or both of these oligonucleotides may be ligated to a common downstream oligo, which is complementary to both CYP21P and CYP21, dependent upon which CYP allele is present as target. The length of the common oligonucleotide and detecting oligonucleotides are adjusted by including a poly-A tail of length 'n' and 'N' respectively, such that each LDR product has a unique length that is at least 2 bases different in size from any other product. The differences in sequence between the CYP21P and CYP21 detecting oligonucleotides are shown in capitals. Full sequences available upon request.

**21-hydroxylase alleles detected by PCR/LDR in a wild-type individual**

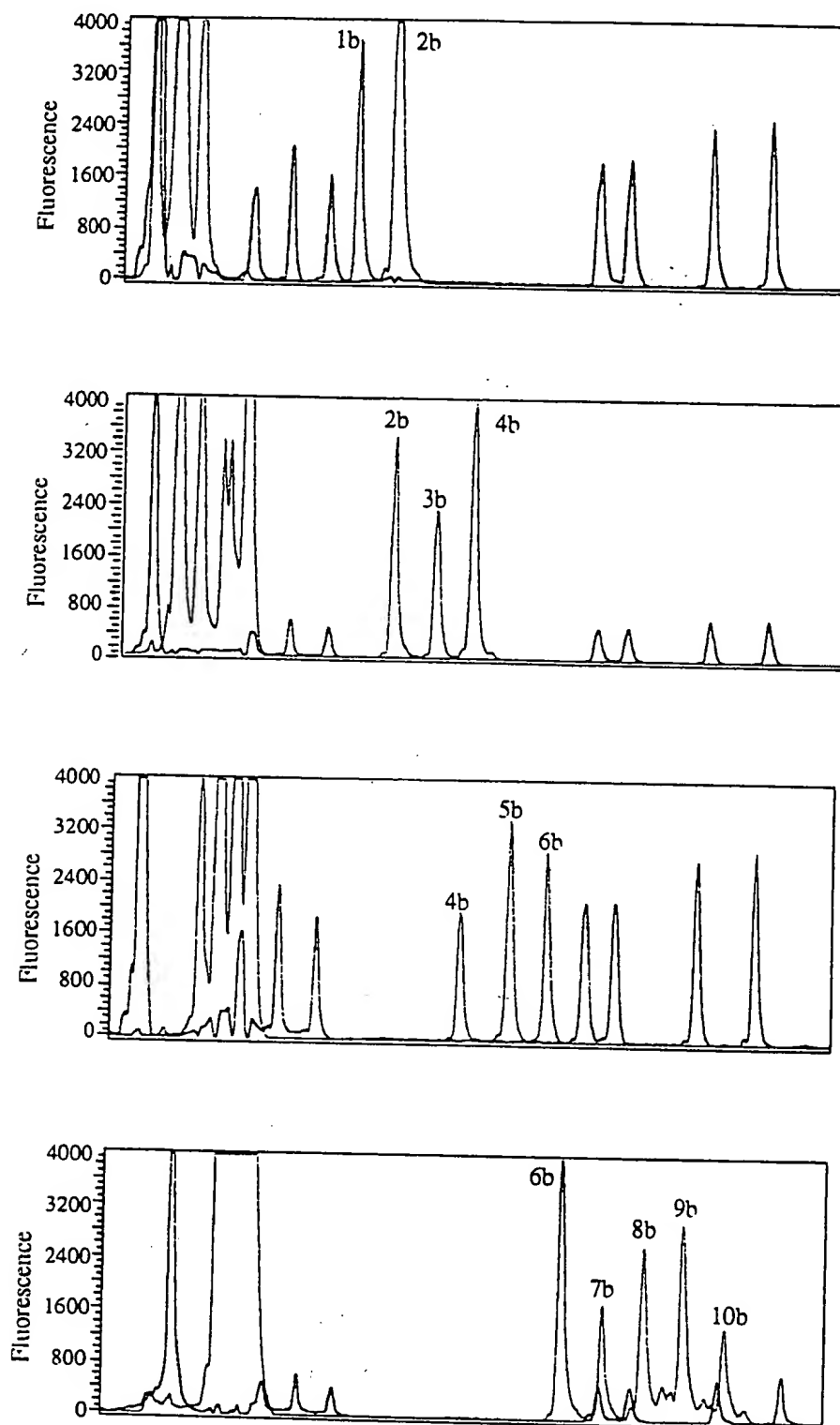
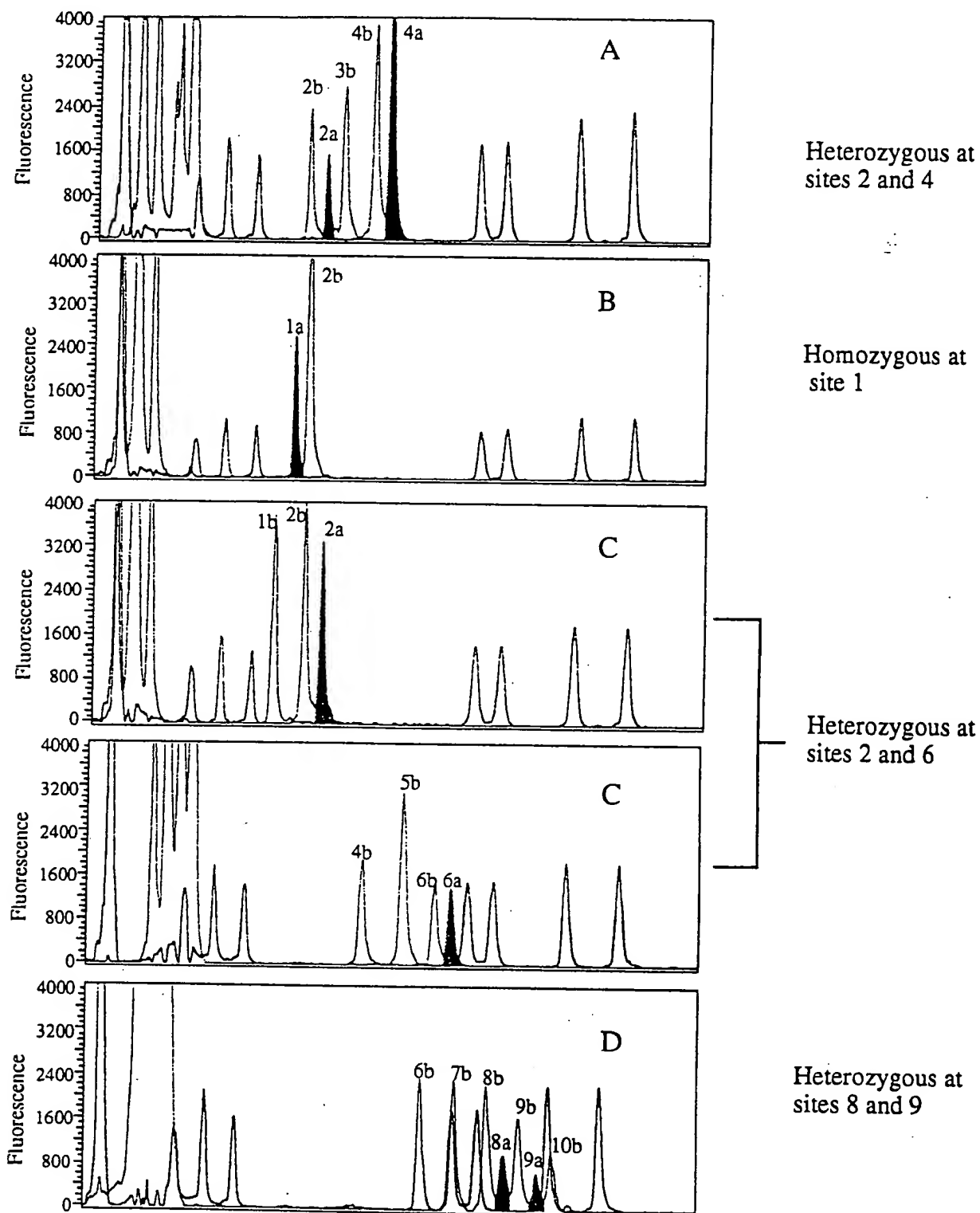


Fig. 7A and 7B. LDR detection *CYP* alleles associated with 21-hydroxylase deficiency. The PCR primers shown adjacent to each panel were used to amplify a segment of the *CYP21* gene from a normal (wild type) individual. The alleles present at each gene conversion site shown in figure 5, were determined using LDR primers specific for both the *CYP21P* and *CYP21* gene. → →

**21-hydroxylase gene conversions detected by PCR/LDR in**  
**21-hydroxylase deficient patients**



(Fig. 7a and 7B, continued) The assigned allele to each LDR signal (blue chromatograms) are labeled wild-type(Wt, *CYP21*) or mutant (Mt, *CYP21P*) and the gene conversion sites are numbered as in figure 5. The LDR signals were assigned according to the size of the products which were calculated relative to the ABI Rox 1000 standard (red chromatograms) by the local Southern method using the ABI Genescan 672 software. Mutant alleles are shaded in blue.



**(ii) Thermostable ligase protein studies:**

(a) *Cloning, overexpression and nucleotide sequence of a thermostable DNA ligase-encoding gene.* The *Thermus thermophilus* (*Tth*) DNA ligase-encoding gene (*ligT*) was cloned in *Escherichia coli* by genetic complementation of a *ligts7* defect in an *E. coli* host. Nucleotide sequence analysis of the gene revealed a single chain of 676 amino acid residues with 47% identity to the *E. coli* ligase. Under *phoA* promoter control, *Tth* ligase was overproduced to greater than 10% of *E. coli* cellular proteins. Adenylated and deadenylated forms of the purified enzyme were distinguished by apparent molecular weights of 81 kDa and 78 kDa, respectively, after separation via sodium dodecyl sulfate-polyacrylamide-gel electrophoresis. Using site directed mutagenesis we have pinpointed active site K118 and D120 residues, which play an important role in adenylation and deadenylation. (J. Luo and F. Barany, unpublished results, For more detail, see Project 4.)

**(iii) Detection of rare point mutations:**

(a) *Development of assays for detecting rare mutations at restriction endonuclease recognition sites.* In collaboration with Dr. Vincent Wilson, a method was developed for detection of point mutations in pre-existing restriction-sites at sensitivities of 1 in  $10^6$  or better (See Project 1, and Flow chart, Fig. 8) The *Ha-ras* gene contains an *MspI* restriction site (CCGG) at the codon 12 locus (CCGGC), such that a mutation in either of the first two bases may be selectively amplified due to the loss of the *MspI* recognition sequence. Three sets of PCR primers were chosen to enable three cycles of nested amplification of mutant alleles with *MspI* selection (See Fig. 9 of *ras* primers). (See similar approaches by [83, 89-95]). An additional level of sensitivity was added by using LCR primers to identify the C → T, or A mutation in the first position of codon 12 (For more detail, please see Project 1). We have termed this method PCR/RE/LCR.

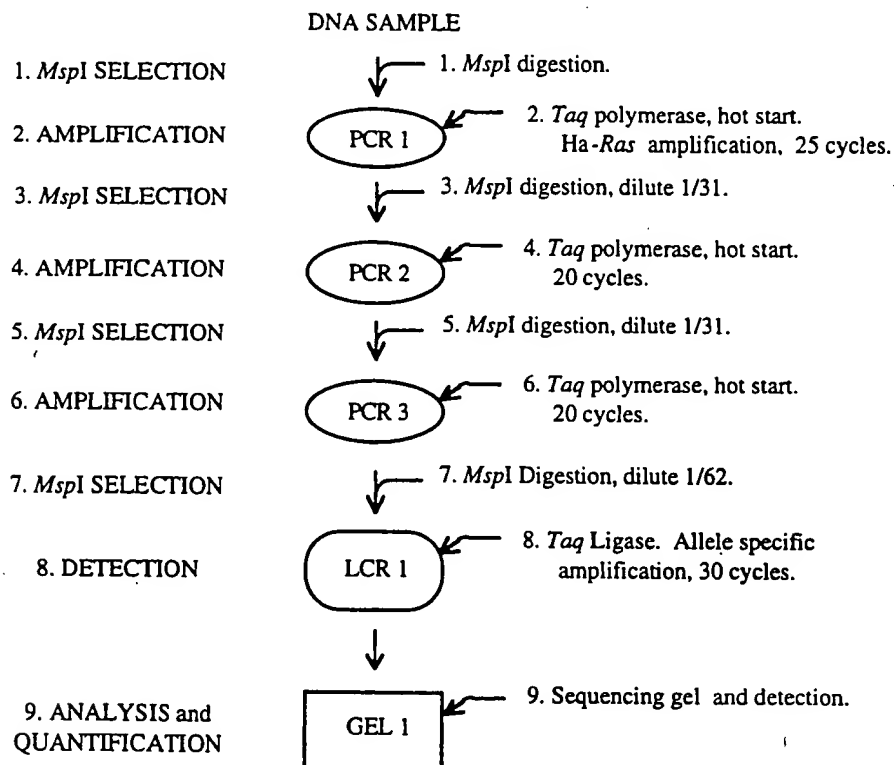
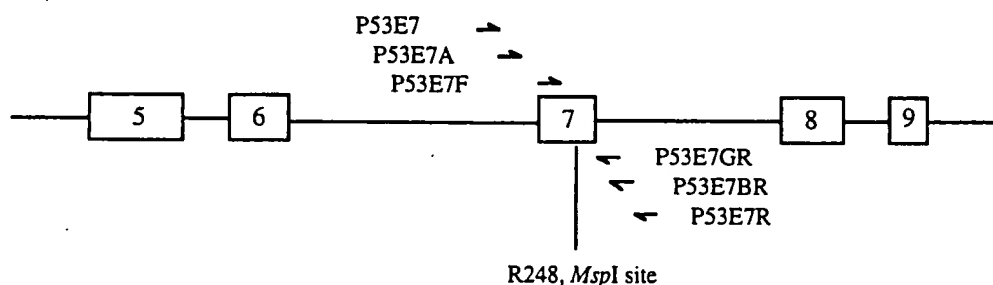


Fig. 8. Diagram of the PCR and restriction enzyme selection of mutations in *Ha-ras* codon 12 and *p53* codon 248. DNA samples are subjected to three cycles of *MspI* restriction and PCR amplification followed by a final *MspI* restriction prior to LCR. The combined total PCR cycles was 65, followed by 30 cycles of LCR amplification. Discriminating primers specific for the wild type and the mutant (G → T) were picked to optimize the specificity of the LCR amplification process according to previously reported studies [54, 55]. The LCR amplification reaction was performed in the presence of radioactive ( $^{32}\text{P}$ ) end-labeled invariant primers, as previously described [54, 55]. LCR products were then separated on a 10% polyacrylamide sequencing gel and detected by exposure of the dried gel to X-ray film. Please see Project 1 for results and primer design.

This selection process enabled the detection of 10 copies of mutant plasmid DNA in the presence of  $10^9$  copies of wild type DNA. This is equivalent to a detection sensitivity of one mutant allele in  $10^8$ . This is at least 5 to  $10^3$  fold better sensitivity than previously reported, indicating the added sensitivity an LCR detection step can provide.

These procedures have also been developed for the human p53 codon 248 (CCGG), which is also within an *MspI* recognition sequence. This first base in codon 248 is a 5-methyldeoxycytidine site and exhibits a high frequency of C  $\rightarrow$  T transition [96, 97, 99-101]. PCR and LCR primers were synthesized and tested on control and mutant DNA (See Fig. 9). Cell mixing experiments of cultured p53 codon 248 wild type cells (LS180) and p53 mutant cells (SW837, CGG  $\rightarrow$  TGG) were performed to determine the practical sensitivity of this technique and the amount of DNA required for maximum efficiency (e.g. 3 ml of blood yields about 60  $\mu$ g of DNA and is equivalent to  $10^7$  cells). The method PCR/RE/LCR method was sensitive enough to detect one mutant cell pelleted into  $10^7$  normal cells (See Project 1, Preliminary results.) These results cleanly demonstrate the exquisite sensitivity of PCR/RE/LCR. This sets the standard for comparing detection sensitivity with our generalized method for converting any CG dinucleotide into a *TaqI* site (see section below.)

### 1. PCR and biochemical selection reactions:



### 2. LCR reaction:

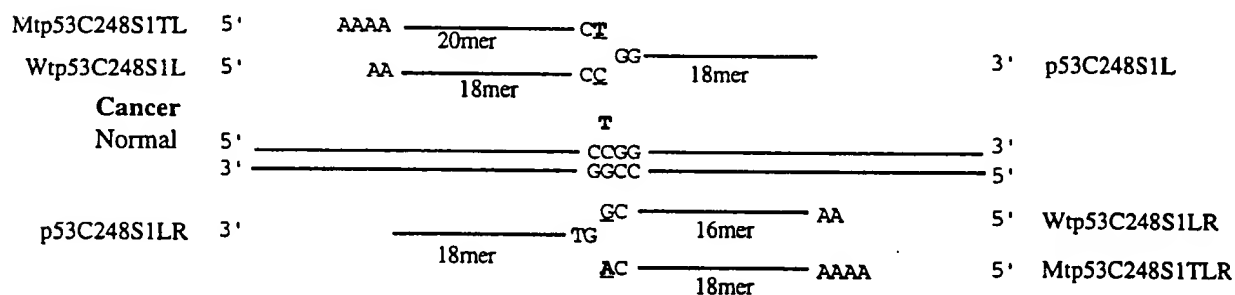
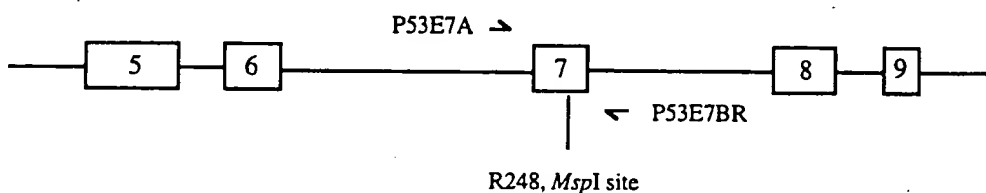


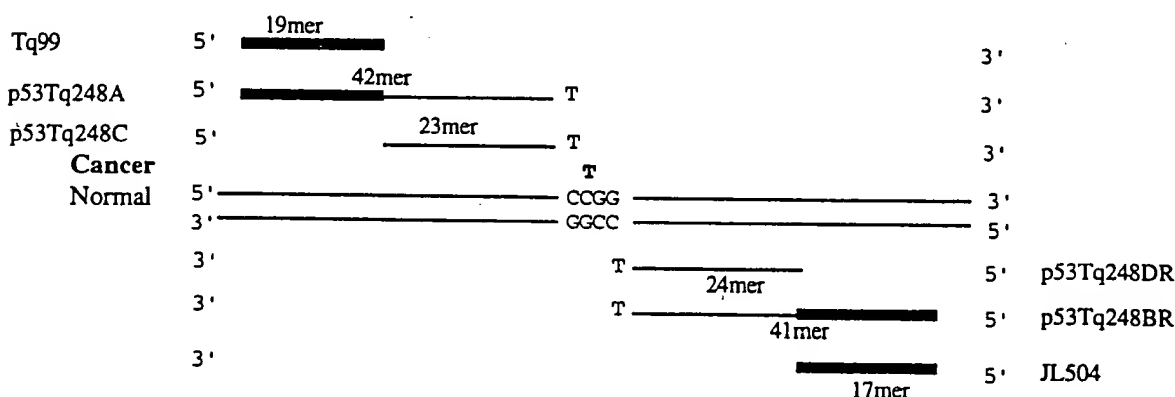
Fig. 9. Diagram of the PCR and LCR primers used for selection of mutations in p53 tumor suppressor gene codon 248. 1. Position of primers. Primers P53E7 and P53E7R specifically amplifies a region of the p53 gene from human genomic DNA. After *MspI* selection, a smaller region is amplified using P53E7A and P53E7BR. By nesting the PCR reaction, one avoids amplifying incorrect amplicons from the first round of PCR. Likewise, primers P53E7F and P53E7GR amplify an even smaller fragment of 240 bases which flanks codon 248. 2. LCR detection of mutant sequences at codon 248. Primers Wtp53C248S1L, and Wtp53C248S1LR are LCR primers whose 3' base is complementary to the wild type p53 codon 248. These primers will amplify using LCR in the presence of adjacent common primers p53C2481L, p53C248S1LR, and wild type target DNA. Primers Mtp53C248S1TL, and Mtp53C248S1TLR are complementary to the R248W mutation (C  $\rightarrow$  T). These primers will ligate to the common primers p53C2481L, p53C248S1LR only in the presence of target DNA containing the R248W mutation. LCR products were then separated on a 10% polyacrylamide sequencing gel and detected by exposure of the dried gel to X-ray film.

(b) *Development of assays for detecting rare mutations at CG dinucleotides by conversion to a *TaqI* recognition site.* Encouraged by our results with PCR/*MspI* selection/LCR detection of one mutant Ha-ras allele in  $10^8$  wild type sequences, primers were designed for converting p53 codon 248 from an *MspI* site to a *TaqI* site (See Fig. 10). These primers were designed to overcome the following two problems which arise from the necessity of having primers separated by only a CG dinucleotide pair: (i) the inability to "nest" PCR primers could result in unwanted primer dimer products; and (ii) an excess of PCR primers overlapping the LDR primers might interfere with ligation. These problems were overcome by designing a shorter set of conversion primers (23 and 24 mer), and a second longer set (41 and 42 mer) containing target sequence and "zip codes" on their 5' ends. The last set of primers contained only the "zip codes" (17 and 19 mer). We have coined the term "zip codes" to refer to primer sequences which have no homology to either the target sequence or other sequences on the genome. "Zip codes" are powerful tools which take advantage of the unique ability of a DNA strand to hybridize only to its complement.

### 1. PCR amplification of p53 Exon 7.



### 2. PCR conversion to *TaqI* site.



### 3. LDR detection of wildtype and mutant sequence.

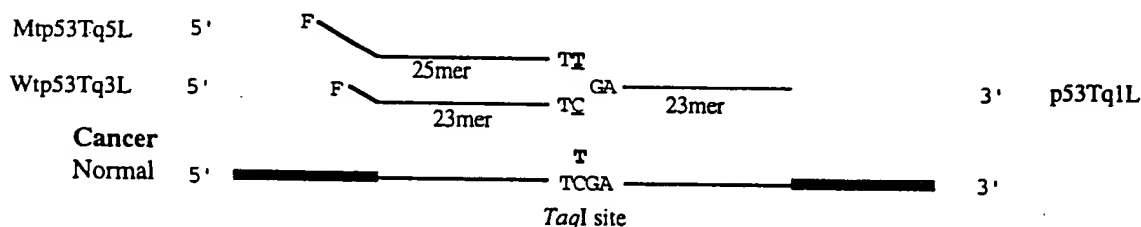


Fig. 10. PCR and LDR primers for high sensitivity detection of mutations in p53 codon 248 using PCR/RE/LDR. The wild type sequence at codon 248 is CCGG, an *MspI* site. For purposes of illustration a cancer-associated, CTGG mutation (R248W) is also shown. 1. Positions of PCR primers P53E7A and P53E7BR. These PCR primers amplify a 240 bp region of p53 containing codon 248 in exon 7. 2. Schematic representation of primers used to convert wild type codon 248 into a *TaqI* site (TCGA). PCR primers p53Tq248C and p53Tq248DR are complementary to the p53 gene and flank the CG dinucleotide in wild type codon 248. Both primers have T:G mismatches at their 3' ends, but are able to PCR amplify in the presence of high dNTP concentrations. Primers p53Tq248A and p53Tq248BR contain the same 3' sequences as p53Tq248C and p53Tq248DR respectively. However, their 5' sequences are identical respectively to "zip code" primers, Tq99 and JL504. "Zip code" sequences have no homology to human genes. Thus primers Tq99 and JL504 will amplify the codon 248 region only if the longer primers have converted the wild type sequence to a *TaqI* site and the R248W sequence to TTGA. Most of the PCR products from wild type sequence can now be eliminated by *TaqI* digestion. See Fig. 11. 3. LDR detection of wild-type and mutant sequences at codon 248. Primers Wtp53Tq3L and p53Tq1L are complementary to the p53 exon 7 sequence, except for the bases flanking the CG sequence of codon

248. These LDR primers will ligate only to wild type target which has been converted to a *TaqI* site (TCGA) at codon 248. Primer Mtp53Tq5L contains a T on its 3' end. It will ligate to p53Tq1L only if the mutant target has been converted to TTGA at codon 248. Primers Wtp53Tq3L and Mtp53Tq5L are fluorescently labeled and differ in length so their ligation products may be distinguished and quantified on an ABI 373A DNA sequencing apparatus.

The general scheme for DNA amplification, site conversion, and *TaqI* selection is shown in Fig. 11. The initial step was to amplify p53 gene exon 7. Codon 248 was converted to a *TaqI* site using the shorter conversion primers for three PCR cycles, followed by addition of the longer primers and two more PCR cycles. After completion of these first five cycles, the PCR reaction was continued by cycling above the  $T_m$  of the shorter conversion primers, and terminated with a short digestion with *TaqI*. These conditions were optimized to convert the majority of codon 248 into a *TaqI* site while minimizing primer dimer formation.

In a second round of amplification using the zip code primers, the amplification reaction was treated twice with *TaqI* endonuclease, cleaving converted wild type sequence while allowing for mutant sequence (and primer dimer) to amplify selectively. A very small percentage of the longer primers from the first amplification round lose their 3' mismatched base, either through exonuclease or exposure to thermal-cycling conditions in the presence of  $Mg^{2+}$ . These truncated primers are far more efficient in extending during amplification. Although present in less than 0.1%, the products thus formed are not converted to a *TaqI* site, and hence amplify as efficiently as cancer mutant DNA. This problem was solved by repeating the *TaqI* conversion amplification, and the "zip code" primer amplification in the absence of *TaqI* selection. These procedures allowed for accurate quantification of mutant and wild-type signal using LDR.

Allelic specific LDR on the above PCR products was performed with fluorescent primers. The products were separated on an ABI 373 DNA sequencer. This method was used to quantify the ratio of wild-type to cancer mutant signal after *TaqI* selection (See Fig. 12). Pure cancer mutant DNA control and pure wild type control gave strong allelic specific signals. The wild-type DNA also showed a small shoulder of mutant signal. The ratio of this large to minute peak represents the ligase specificity (about 50:1), which will be further improved as described in the Methods section (iib). The next chromatogram demonstrates that primer dimer (generated separately from a control tube without DNA template) gives no signal with LDR detection, consistent with the specificity of LDR. The bottom three chromatograms show wild type DNA containing zero,  $10^{-3}$ , and  $10^{-5}$  dilutions of DNA containing the cancer mutation which have undergone this *TaqI* selection procedure. The ratio of the two peaks indicates a stronger signal for the cancer mutation at both 1 in  $10^5$  and especially at 1 in  $10^3$  dilutions (F. Barany and D. Day, unpublished results).

Fig. 11. Flow chart for conversion of p53 codon 248 to a *TaqI* site by PCR/RE/LDR (See following page). A 240 bp region containing codon 248 in exon 7 of p53 was amplified. DNA samples were prepared by serially diluting mutant DNA product into wild type product to simulate the presence of 0 cancer cells (no dilution control), 1 cancer cell in  $10^5$  normal cells and 1 in  $10^3$ . I. Wild type and mutant sequences in exon 7 were amplified by PCR. II. Wild type codon 248 was converted to a *TaqI* site using the shorter conversion primers (p53Tq248C and p53Tq248DR) in three PCR cycles of 94°C, 30"; 60°C, 2', followed by addition of the longer primers (p53Tq248A and p53Tq248BR) and two more PCR cycles under the same conditions. After completion of these first five cycles, the PCR reaction was continued by 12 cycles above the  $T_m$  of the shorter conversion primers (94°C, 15"; 75°C, 30"), and terminated with a short digestion with *TaqI*. The 12 higher temperature cycles are referred to as product only cycles, since the primers do not hybridize to the native p53 target at these higher cycling temperatures. These conditions also minimize primer dimer formation. III. & IV. A second round of amplification used the zip code primers (Tq99 and JL504) for 30 cycles (94°C, 15"; 55°C, 30", 65°C 1') and two additions of 100U *TaqI* endonuclease to cleave converted wild type sequence while allowing mutant sequence (and primer dimer) to amplify selectively. The majority of "primer dimer" resulted from incomplete *TaqI* site conversions due to amplification of truncated primers which were missing the 3' T:G mismatch. These could be converted into *TaqI* sites by repeating steps 2-8. V. *TaqI* digestion was not included in the final zip code amplification so the remaining undigested wild type sequence could serve as an internal control. VI. PCR products were diluted 20 fold, *Taq* polymerase was inactivated, and 2 cycles of LDR were performed (94°C, 30"; 64°C 5') using the common primer p53Tq1L and fluorescently labeled primers Wtp53Tq3L and Mtp53Tq5L. VII. Products were separated by electrophoresis on an ABI 373A DNA sequencing apparatus and quantified using the Genescan 672 software.

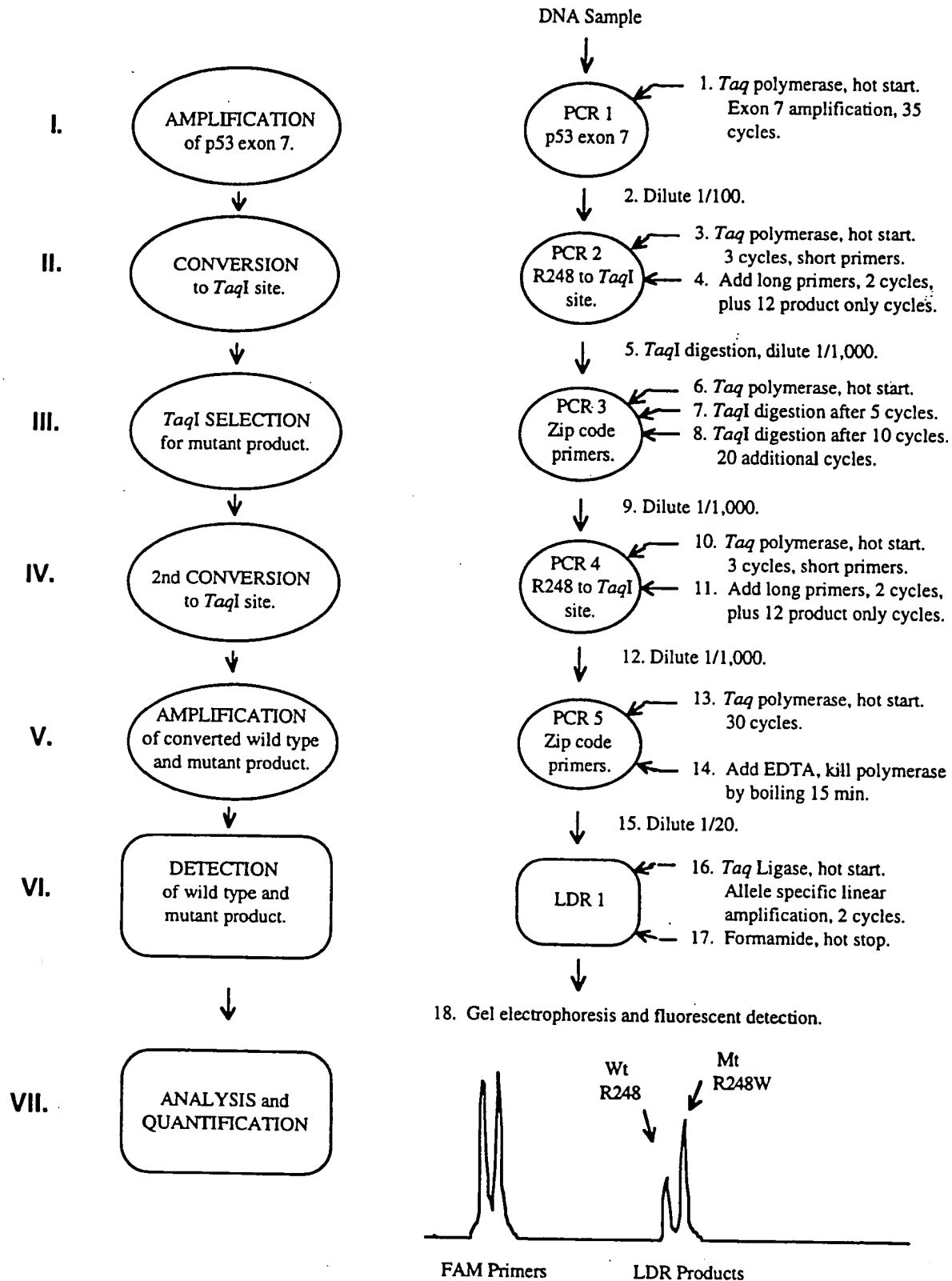


Fig. 11. Flow chart for conversion of p53 codon 248 to a *TaqI* site by PCR/RE/LDR (See previous page for Legend).

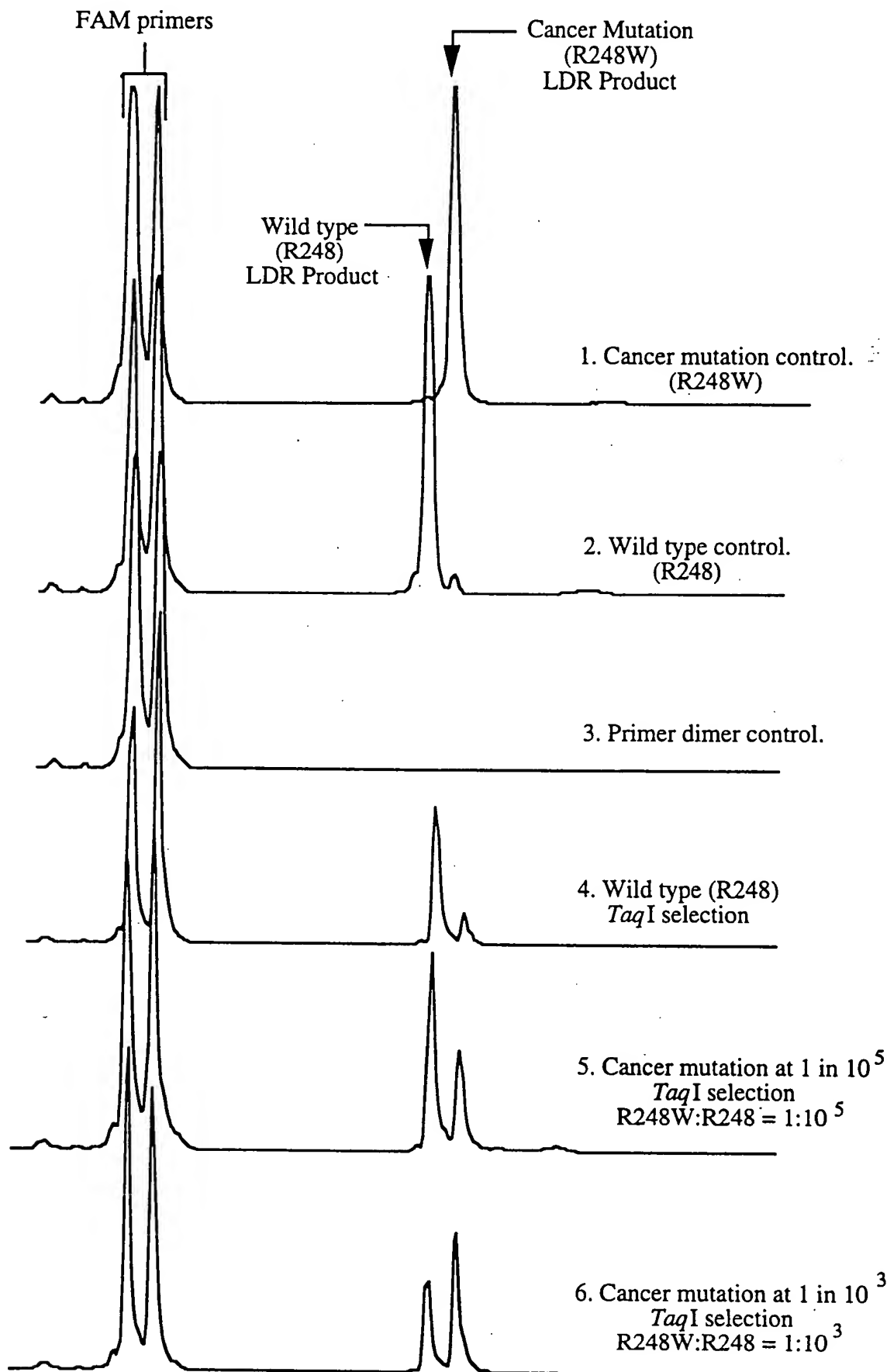


Fig. 12. Detection of p53 R248W mutation at 1 in  $10^3$  and 1 in  $10^5$ . (See following page for legend.)

Fig. 12. (See previous page). Detection of p53 R248W mutation at 1 in  $10^3$  and 1 in  $10^5$ . Chromatograms of p53 LDR products separated on an ABI 373A DNA sequencer. Fluorescently labeled primers are 23 (wild type) and 25 (mutant) bases long; products are 46 and 48 bases respectively. Target DNAs: 1. Control p53 R248W mutant DNA converted to a TTGA sequence. 2. Control p53 R248 wild type DNA converted to a *TaqI* sequence TCGA, without *TaqI* selection. 3. Control primer dimer target generated by PCR amplification of longer primers in the absence of p53 target. 4. Wild type p53 R248 DNA converted to a *TaqI* sequence, with *TaqI* selection. 5. Wild type p53 R248 DNA containing 1 in  $10^3$  R248W mutant DNA converted to a *TaqI* sequence, with *TaqI* selection. 6. Wild type p53 R248 DNA containing 1 in  $10^5$  R248W mutant DNA converted to a *TaqI* sequence, with *TaqI* selection. The ratio of the mutant and wild type peaks in a chromatogram reflects the relative amount of mutant DNA present in the original sample to an order of magnitude.

## D. EXPERIMENTAL DESIGN AND METHODS

It has been hypothesized that multiple mutations in cellular growth- and differentiation-regulating genes are responsible for the development of cancers [116]. A model for the development of colon cancers is generally accepted [9] and a model for breast cancer carcinogenesis has been proposed [117]. Our purpose is to simultaneously characterize as many of these mutations as possible. This may lead to more accurate prognosis and more appropriate treatment of breast cancers. Identification of viral sequences in HPV infections will allow for more accurate prognosis and treatment of those at high risk for development of cervical or vaginal cancers. To achieve our purpose we aim to develop three technologies:

- (i) *a multiplex polymerase chain reaction/ligase detection reaction (PCR/LDR) system for detection of point mutations in tumor biopsies.* Expected result: Rapid and simultaneous detection of 24 to 40 point mutations, representing from 63% to 79% of mutations, in the p53 gene from breast tumor specimens. Rapid and simultaneous detection of high risk HPV in cervical lavages or biopsies.
- (ii) *a ligase detection reaction/polymerase chain reaction (LDR/PCR) system for detection of gene amplifications and deletions in tumor biopsies.* Expected result: Rapid and simultaneous detection of HER-2/neu and int-2 amplifications and deletion of p53 in breast tumor specimens.
- (iii) *a polymerase chain reaction/restriction endonuclease/ligase detection reaction (PCR/RE/LDR) to detect and identify mutations at a sensitivity of 1 in  $10^6$  or  $10^7$  cells.* Expected result: Detection of micrometastases to lymph nodes and bone marrow.

The principal investigator and co-investigator consider ourselves fortunate to be able to collaborate with Dr. John Kovach, MD, Chairman of the Division of Oncology and his associate, Dr. Steven Sommer, MD/Ph.D., Professor of Molecular Biology at Mayo Clinic, Dr. Thierry Soussi, Ph.D., Professor of Biochemistry, Institute of Molecular Genetics at the Pierre and Marie Curie University, France, Dr. Michael P. Osborne, MD chief of the Division of Breast Surgery at the New York Hospital-Cornell Medical Center, and Dr. Saul Silverstein, Ph.D., Professor and Acting Chairman of Microbiology, College of Physicians and Surgeons of Columbia University, New York. (Please see letters of collaboration). Drs. Kovach and Summers have offered to provide us with touch preps and amplified DNA from tumors with various defined p53 mutations as well as uninvolved lymph nodes from the same individuals. Dr. Soussi has provided us with the most comprehensive data base on p53 mutations in all cancers, numbering over 2,000 cases, as well as access to tumor samples of known p53 mutation. Dr. Osborne has offered us access to several breast cancer cell lines and over 600 bone marrow samples from women with breast cancer and their corresponding tumors and lymph nodes. Many of these tumors have been characterized with regard to HER-2/neu gene amplification and conventional pathological parameters. Dr. Silverstein will provide both DNA and clinical cervical tissue samples which have already been typed by earlier methods.

(i) **Development of a multiplex polymerase chain reaction/ligase detection reaction (PCR/LDR) system for detection of point mutations in tumor biopsies.** Our laboratory has developed a PCR/LDR method for multiplex discrimination of ten gene conversion mutations which cause 21 hydroxylase deficiency. This PCR/LDR technology will be extended to identify cancer causing mutations in

the p53 tumor suppressor gene in a variety of characterized cell lines and "touch prep" DNA samples. By optimizing ligation conditions and/or using mutant *Tth* ligase, we aim to increase the sensitivity of this assay to detect one cancer gene mutation in  $10^2$  to  $10^3$  normal cells (See Project 4). We plan to develop this assay to simultaneously detect possible mutations in any one of 24 to 40 codons in the p53 gene in frozen and fixed primary breast tumors from the 100 to 200 breast cancer cases. The PCR/LDR technology will also be used to type HPV strains in coloscopy samples which correlate with cervical cancers. Oligonucleotide or PNA addressable arrays will allow for simultaneous screening of dozens to hundreds of potential mutations (See Project 5 and Core 2).

(a) *Multiplex PCR/LDR for detection of 24-40 mutations in p53 exons 5, 6, 7, and 8.* The general strategy for simultaneous PCR/LDR detection of multiple mutations is shown in Fig. 13. Primer pairs or nested primer pairs will be used to amplify p53 exons 5 & 6, 7, and 8 [10, 26, 118]. Initially, DNA prepared from known cell lines or sequenced tumor samples will be used to validate the sensitivity and specificity of detecting a given mutation. LDR primers will be synthesized for the most commonly reported breast cancer single base changes in a given exon (185 mutations compiled from refs. [10, 23, 26, 118-123]). The initial five codons to be tested are V157 (2.2%), R175 (10.7%), R248 (12.4%), R273 (7.3%), and R282 (5.6%), which account for 23% of the reported mutations. These codons are chosen because they represent common mutations at CG dinucleotide sites, which may be converted to *TaqI* sites for detection of micro metastases (see Specific aim (iii)). Inclusion of codons Q136, P152, Y163, K164, H179, R181, G187, L194, L201, R213, M237, C238, C245, R249, I254, L265, R280, D281 and E285 brings the total codons to 24 and covers 63% of known mutations. Inclusion of the top 40 codons would cover 79% of reported p53 mutations in breast cancer samples. For the purposes of this pilot project, we will synthesize 12, 6, and 6 LDR primer sets for amplified p53 exons 5 & 6, 7, and 8 respectively (see Fig. 13). Each LDR primer set contains two different length fluorescently labeled oligonucleotides which contain the discriminating bases on the 3' ends (see Fig. 10. part 3 for diagram of sample primers.) When more than one mutation has been reported for a codon, such as codon 248, additional fluorescent primers with the discriminating base on the 3' end will be used. Ligation of one of these discriminating oligonucleotides to an adjacent common oligonucleotide will generate a product corresponding to either mutant or wild-type allele. LDR primers will be designed to have a  $T_m$  of about 65°C with the aid of Oligo 4.0 software. The common oligonucleotides will contain poly A or hexaethylene oxide "tails" of increasing length on their 3' ends to allow for separation and detection of each product set on DNA sequencing gels [54, 55, 78]. Signals will be quantified using the Genescan 672 software. Alternatively, when the addressable array is synthesized and tested (see Project 5), LDR primers will have "zip code" tails, and signal distinguished by position of fluorescent signal on the array. Signal would be quantified using a Molecular Dynamics FluorImager (See Core A and B). Even when LDR primers overlap in the detection of mutations at adjacent codons overlap, they do not interfere with the proper ligation reaction. In addition, all LDR primers are on the same strand to avoid one primer serving as a bridging template for two others. We have already employed this strategy for simultaneous LDR identification of 21 hydroxylase deficiency alleles; 30 primers are mixed in a single tube, allowing for discrimination of 20 different products (D. Day, P. White, and F. Barany, unpublished results). Initial experiments will use tissue or DNA samples where the p53 mutation has already been determined by DNA sequence analysis. As each primer set is validated, it will be added to the reaction mixture primer pool. Subsequently, this technique will be tested with unknown samples derived from touch preparations which are generally homogenous for p53 mutations [10, 118-120]. See section (c) below.

(b) *Improving the sensitivity of PCR/LDR mutation detection.* One of the key issues in any mutation detection scheme is the level of sensitivity. DNA prepared from cell lines or "touch" preps tend to be homogeneous. Thus a clean mutant signal is observed on a sequencing or SSCP gel. However, when the mutant signal is less than 25% of the total signal, it may no longer be distinguishable from background. In contrast, detection of an LDR signal is dependent on the fidelity of *Tth* ligase. Initial experiments in the P.I.'s laboratory demonstrated the exquisite fidelity of *Tth* ligase. The enzyme demonstrated a 50 fold discrimination between an A:T match and a G:T mismatch, and greater than 500 fold between an A:T match and an A:A mismatch at the ligation junction in an LDR reaction [54]. Our aim is to develop a rapid and more sensitive assay for systematically measuring the fidelity of wild-type and mutant *Tth* ligases. Optimizing conditions and/or use of mutant enzymes may increase the sensitivity of direct PCR/LDR detection of mutations in the 1 in  $10^2$  to 1 in  $10^3$  cell range. This approach is discussed in greater detail in Project 4.



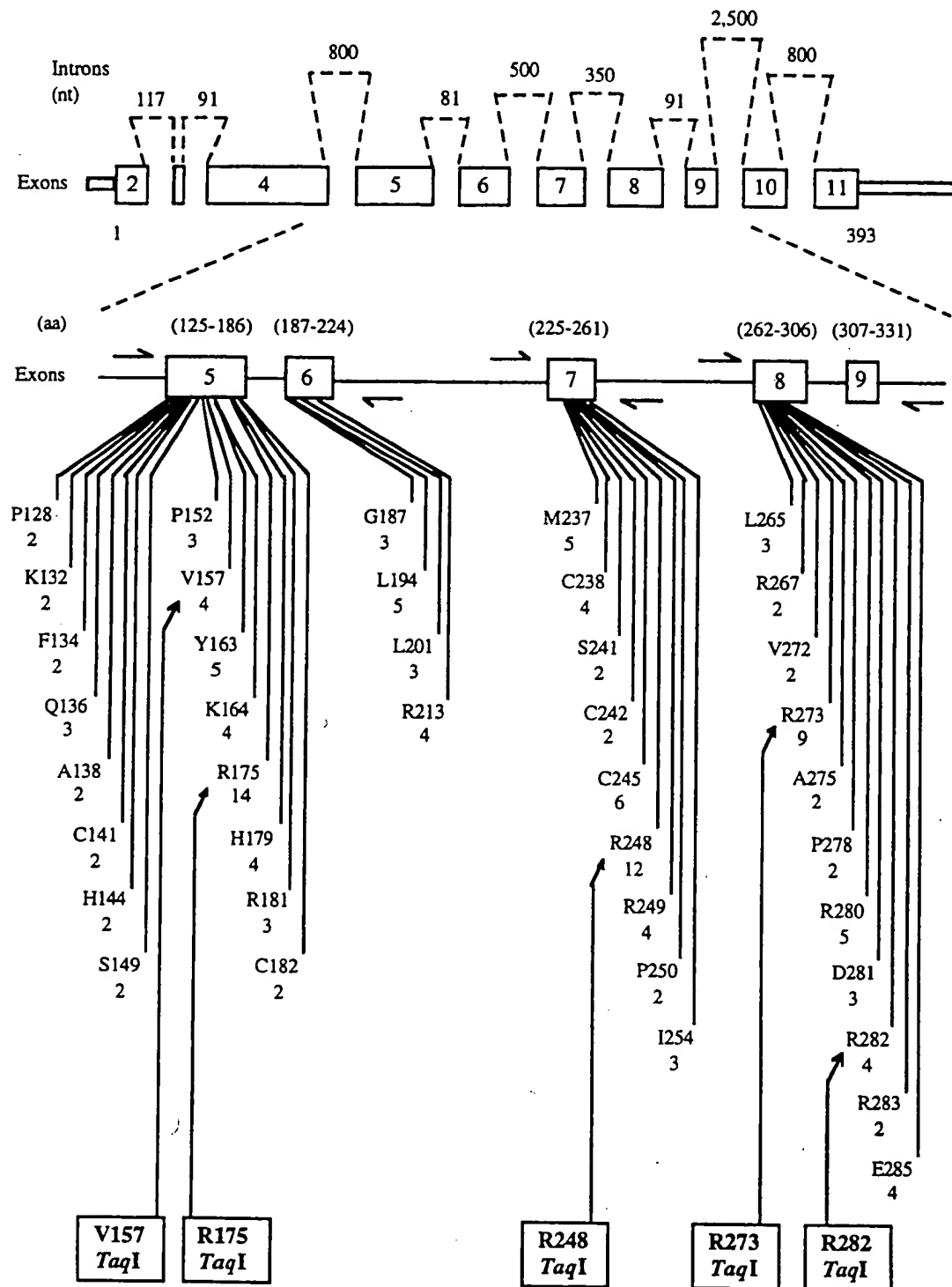


Fig. 13. Positions of the most common p53 mutations in breast cancer. This schematic of the p53 gene shows the approximate length and size of p53 exons and introns. Regions from exons 5 through 9 corresponding to amino acid residues 125 to 331 are shown in an expanded view. More than 95% of p53 point mutations have been found in these exons [52]. Horizontal arrows flanking the exons represent PCR primers (or nested primers if needed) for initial amplification of exons 5 & 6, 7, and 8. The positions of the 40 most common mutations are indicated, with the number of mutations detected shown below that codon. This represents 79% of 185 reported p53 mutations [119, 120]. Initially, LDR primer sets will be designed and synthesized for codons V157, R175, R248, R273, and R282. These positions represent common mutations at CG dinucleotides and may be converted into *TaqI* sites for PCR/RE/LDR detection of 1 mutation per  $10^5$  to  $10^7$  cells. Lymph node and bone marrow tissue of patients with primary breast tumors containing these five mutations will be analyzed by PCR/RE/LDR for evidence of micrometastases. The initial LDR primer set will be expanded to include all codons with 3 or more mutations (24 codons or 63% of mutations shown in this figure.)

(c) *Detection of p53 point mutations in breast tumor samples.* When PCR/LDR detection for codons V157, R175, R248, R273 and R282 are feasible, we will begin to apply the technique to our set of 100 to 200 primary tumor samples. (We will search lymph node and bone marrow specimens for this set of mutations. See Methods section iii.) Eventually we will expand the number of codons at which we can detect mutations to 24 to 40. DNA will be extracted from the specimens and simultaneous detection of mutations in these codons will be performed as described above. DNA sequencing on a small number of tumors will be performed to confirm the accuracy of the method. As a further check on the validity of the assay, the frequency of mutations discovered by PCR/LDR will be compared to mutation frequencies found in previously published studies [10, 23, 26, 118, 121-123] and databases of p53 mutations in breast cancer [119, 120]. Finally, many of these tumors have been characterized for HER-2/neu amplification and the presence of bone marrow micrometastases by immunohistochemical staining. The types of mutations we find will be correlated with these laboratory parameters, the results of our studies of HER-2/neu and int-2 amplification in this tumor set (See Methods section ii.) and clinical prognostic parameters (e.g. primary tumor size, lymph node status and estrogen receptor status).

An important aspect of this work will be to define the sensitivity of PCR/LDR assays. With current enzyme and ligation conditions, our assay has a sensitivity of 1 in  $10^2$ . When identifying mutations from clinical samples, we will only use mutation-specific primers to avoid excessive wild type signal. We can include an external control sequence containing an artificially introduced marker mutation (such as R248G, see Fig. 14 for details) to quantify the amount of mutation in the sample. This method may also identify microheterogeneity, as subpopulations of cells may have acquired secondary mutations.

If a particular mutation is identified in a primary tumor, would PCR/LDR be sufficiently sensitive to search for micrometastases in lymph nodes? Would 1 in  $10^3$  or 1 in  $10^4$  sensitivity be sufficient? We have isolated *Tth* ligase mutants and designed internally mismatched LDR primers with the aim to improve both sensitivity and specificity of PCR/LDR (See Project 4). Should these methods provide the exquisite specificity but fail to generate sufficient LDR product signal (i.e. insufficient sensitivity), we could synthesize a set of LCR primers, complementary to the LDR primers. The sensitivity of the PCR/LDR reaction could then be extended orders of magnitude by a subsequent LCR step achieving multiplex PCR/LDR/LCR detection of p53 mutations. The limits of sensitivity of PCR/LDR/LCR are unknown. We have developed PCR/RE/LDR for high sensitivity detection of mutations at a level of 1 in  $10^5$  to  $10^7$  (see Methods section iii). PCR/LDR/LCR may provide an easier method of achieving sensitive and specific detection of mutations from clinical specimens at levels which have clinical significance.

Addressable array capture will eventually be the preferred method of identifying mutations. In our initial examination of the p53 gene in 100 to 200 breast tumor samples we will only look for five different mutations, V157, R175, R248, R273 and R282. By synthesizing LDR primers with tails of varying lengths we can easily distinguish between these mutations using gel or capillary electrophoresis. However, increasing the number of assayable mutations to 40, which represent 79% of p53 mutations identified for breast cancer, eventually makes electrophoretic detection less feasible. This is due to two reasons. First, mutation-specific LDR primers should differ in length by two bases for their products to be distinguished by electrophoresis. For 40 mutations to be assayed together, very long primers would have to be synthesized. Second, only one mutant signal would be expected for most reactions. Since mutant signals will differ from each other by only two bases, minor defects in a gel lane could cause a misreading of the LDR product length and incorrect identification of the mutation. Both of these problems are currently overcome in our laboratory by using more than one color fluorescent label and internal standards within the same lane. However, once the primers become very long (75-100 bases), failure sequences (n-1, n-2) become increasingly harder to separate by HPLC or gel purification. In contrast, by synthesizing a unique 24 base zip code sequence to each LDR primer, the product can be captured by its complementary zip code at a discrete "address" on a two-dimensional array (See Project 5). Failure sequences do not present a problem for either the zip code sequence or its complementary address. A fluorescent signal at a specific address, as opposed to a specific size, thus indicates the presence of a specific cancer mutation. (See Core B.)

(d) *Detection of high risk HPV strains correlated with cervical carcinomas.* There are several methods currently available for detecting the presence of HPV genomes. However, they are technically difficult, time consuming, and of insufficient specificity or sensitivity for detecting minor populations of high risk virus in mixed populations. Commercially available HPV detection systems (ViraPap detection system, and ViraType, Life Technologies Inc., Gaithersburg, Md) have proved to be less sensitive and discriminatory than the PCR methods developed by Manos and co-workers [41, 124]. These workers used PCR consensus primers designed to amplify a 450 bp region of the highly conserved L1 gene that encodes a viral capsid protein. Amplified product was typed by Southern blotting using type specific probes. This method has been further developed by using RFLP analysis of amplified product [44]. Typing by PCR with Southern blot analysis and PCR-RFLP analysis correlate well except for specimens containing multiple HPV types or novel isolates. Such samples may cause problems because of disproportionate PCR amplification of one viral type in a mixed specimen.

The task of HPV typing is exceedingly well suited for PCR/LDR since the ability of LDR to detect single base pair differences enables differentiation of closely related HPV types. This is particularly relevant with HPV where the clinically important question is; "Does the patient have a high risk HPV type infection?" rather than, "What is the HPV type?" Molecular typing allows us to take this one step further and ask: "Does the HPV infection contain the alleles associated with malignant growth?" By testing for the alleles associated with malignancy one not only identifies the high risk types but also identifies new isolates which are potentially oncogenic by virtue of the E6 or E7 allele they possess.

We are proposing an alternative PCR strategy which may overcome the limitations associated with mixed HPV type infections. Rather than using consensus primers to amplify a highly conserved region of the HPV genome, we plan to use the natural deletions, insertions and sequence divergence that have occurred in the E6 and E7 genes to specifically amplify viral types. Analysis of the E6 and E7 ORFs sequences reveal high sequence divergence at the carboxy and amino terminus of both proteins due to deletions and insertions. Such natural sequence variance can be used to distinguish the high risk types by designing PCR primers whose 3' end are complementary to the unique insertions/deletions which allow for allele specific PCR amplifications. Preliminary work by Dr. Octavian Lungu and Dr. Saul Silverstein (personal communication, see also letter of collaboration) has already successfully used a strategy of PCR followed by Southern hybridization to amplify and type portions of the E6 gene. In a collaborative effort, we intend to expand upon this approach by using allele specific PCR to amplify high risk E6 and E7 sequences and then type the amplified products by LDR (See Fig. 14 and Table 1).

Table 1.

PAP01	GTATTGCATTGGAACCTCAG	HPV-45 Forward
PAP02	GAGCCCCAAAATGAAATTCCG	HPV-18 Forward
PAP03R	GCCGTCACACTTACAACATAC	HPV-45 Reverse
PAP04R	GTACCTTCTGGATCCGCCAT	Common Reverse
PAP05	TATGTCTTGTTGCAGATCATC	HPV-16 Forward
PAP06	TTGCATAGCATGTTGGAGAA	HPV-31 Forward
PAP07	GTGTTGGAGGTCCCGACGTAG	HPV-33 Forward
PAP08	ATGCACCAAAAGAGAACTGC	HPV-16 Forward
PAP09	AAAAAAAAATAGGGTGTAACCGAAA	Common Forward
PAP10R	TTCTCTACGTGTTCTTGATGAT	HPV-16 Reverse
PAP11R	CGTTCCTGTCGTGCTCGGTTG	HPV-18 Reverse
PAP12R	GGGTTTCAGTACGAGGTCTTC	HPV-31 Reverse
PAP13R	GCAGTTTCTCTACGTCGGGACC	HPV-33 Reverse
PAP14R	CTTCTTGCCGTGCCTGGTCA	HPV-45 Reverse

Table 1. High risk HPV type specific primers. Sequences of the common primers and the type specific primers are given. The first round of PCR amplification uses the common reverse and forward primers which amplify all of the high risk HPVs and some of the low risk. The second round of amplification uses a semi-nested PCR where a type specific primer is used along with the appropriate common primer.

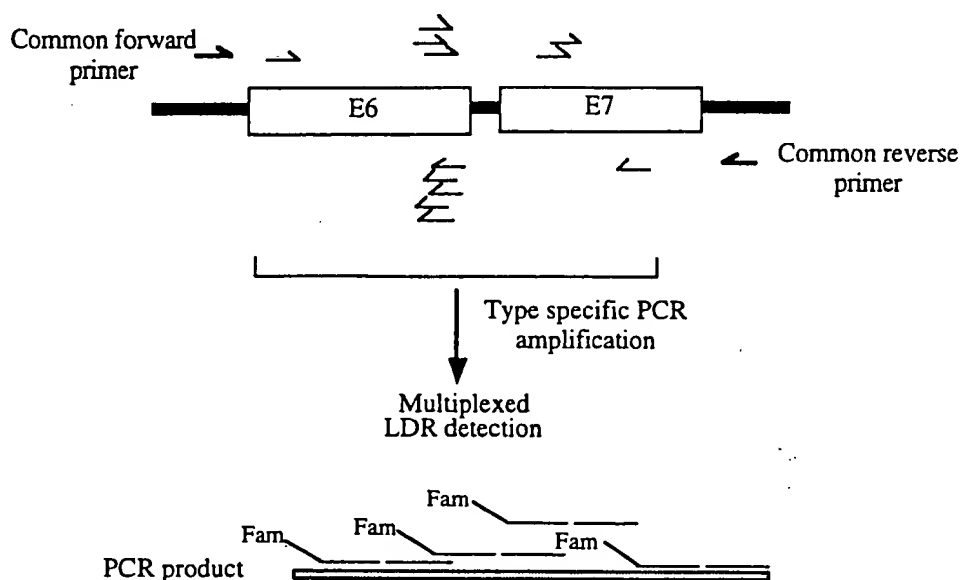


Fig. 14. Detection of High Risk HPV types by type specific PCR-LDR of the oncogenic E6 and E7 genes. The entire E6 and E7 genes are amplified using the common primers and then type specific amplification is achieved using a semi-nested second round of PCR to amplify each high risk type using the specific high risk primers (Table 1). The amplified product is then analyzed in a multiplexed LDR reaction using fluorescently labeled primers that probe discriminating regions of the E6 and E7 genes to type the PCR product. If ligated the LDR oligonucleotides form unique sized fluorescent products which can be differentiated and quantified using an Applied Biosystems Inc. 373A DNA sequencer using Genescan 672 software.

PCR alone is not sufficient for screening for high risk viral types due to the multitude of HPV types already known and novel ones which are continually being discovered. Any supposedly allele specific amplification runs the risk of amplifying low risk sequences as well as high risk, necessitating the use of a second screen such as Southern hybridization or LDR. We have designed PCR primers that can be used to amplify the high risk E6 and/or E7 alleles. Each high risk allele can be amplified and differentiated from the other high risk alleles by a unique PCR primer combination. The amplified product can then be confirmed to be the suspected high risk allele (as opposed to a low risk allele that shares sequence similarity at the PCR primer binding sites) by using multiple LDR primer sets to look for conserved discriminating nucleotides within that allele. The presence of all discriminating nucleotides, will confirm that the amplified product is a high risk allele.

One potential problem in DNA detection of viral sequences is the possibility of polymorphisms surrounding the discriminating nucleotide. We have developed a ligase fidelity assay to test the effect of additional mismatches near the discriminating base on both ligation efficiency and fidelity (See project 4.) One solution is use of the  $Q_2$  nucleotide analogue (3-Nitropyrole deoxyribonucleoside, See project 3) at positions of sequence polymorphisms. This analogue is a "universal" nucleotide analogue, with the ability to base pair to all bases, while allowing for sequence specific hybridization of the neighboring bases in the primer (See preliminary results, Project 3.) We will incorporate the  $Q_2$  nucleotide analogue into both LDR and PCR primers at regions of polymorphism near the discriminating base. This will help assure that the assay is not missing high risk HPV viruses due to nearby polymorphisms.

Use of LDR as the typing method offers several major advantages over Southern blotting and RFLP analysis. The ability of LDR to detect single base pair differences, enables differentiation of closely related HPV types. Infection with more than one HPV type or a closely related type poses problems with the PCR-RFLP and PCR-hybridization analysis currently used. Mixed infection with more than one HPV type would not be problematic in our approach since we have *two* levels of specificity for the high risk HPV types: (i) allelic specific PCR and (ii) multiplexed LDR. Only if both criteria are met will the sample be identified as a high risk HPV. While Southern analysis using a type specific probe on a more selective PCR amplification, such as we have proposed, would also suffice, Southern blotting can not be multiplexed to examine multiple

sites as with LDR. Neither RFLP or Southern analysis compares with respect to specificity, speed, or ease of use to LDR.

Once we have our PCR/LDR technique operational in HPV typing, we can adapt the technique to addressing biological questions concerning E6 and E7 gene product expression. E6 or E7 mRNA may be reverse transcribed. This product can then be detected by PCR-LDR. This will help address the question of whose genes are transcribed during mixed infections. Does infection with a low risk HPV compete with high risk HPV for transcription and translation possibly reducing the amounts of the oncogenic proteins translated? Are the oncogenic E6 and E7 genes always transcribed at the same levels or are they induced to a potentially higher level by environmental factors? By performing RT-PCR-LDR we could develop a functional assay that could directly determine if the oncogenic E6 and E7 genes were transcribed. Such information would be invaluable for the testing of drugs that aim to inhibit the transcription of viral genes. RT-PCR-LDR may have far reaching implications in terms of assaying gene transcription, or in the typing of retroviruses such as HIV.

**(ii) Development of a multiplex ligase detection reaction/polymerase chain reaction (LDR/PCR) system for detection of gene amplifications and deletions in tumor biopsies.** This study aims to develop a technology to investigate the amplification and deletion of several genes in a single assay. We have designed ligase detection primers for the following genes: SOD (on chromosome 21q), G6PD (on chromosome Xq), p53 tumor suppressor gene (on chromosome 17p), HER-2/neu (on chromosome 17q), and Int-2 (on chromosome 11q). Each set of LDR primers includes the same external "zip code" sequences, which will allow for proportional amplification of all five chromosome probes with a single pair of PCR primers. The method will be validated with genomic DNA samples from normal, and trisomy 21 human males and females. Subsequently, cell lines with known HER-2/neu and int-2 gene amplifications, and p53 deletions will be used to test the sensitivity of this technique. The assay will be extended to frozen and fixed breast tumor material. Initial studies will investigate the 100 to 200 tumors to determine the feasibility of large scale studies correlating multiple genetic alterations to the clinical/biological behavior of breast cancers.

We will use the Ligase Detection Reaction (LDR) in the first step of gene quantification to create artificial PCR templates (see Fig. 15). The LDR products will be in the same ratio as the genes from which they were derived, but will also have the same artificial PCR primer sequences (which we call "zip code" sequences) at their ends. Using only two PCR zip code primers, all of the LDR templates will be amplified together, maintaining proportionality. We will fluorescently label one of the zip code primers to allow quantification on a DNA sequencing apparatus. Because the LDR templates will include a restriction enzyme site at a unique position, restriction digestion of the PCR products will permit us to distinguish the products from different genes on the basis of their lengths. We will first test the technology by identifying the dosages of genes on chromosomes X and 21 in male, female and trisomy 21 DNA's. Investigations of deletion of p53, and amplification of HER-/neu and int-2 in breast tumors and cell lines will follow.

**(a) Design, synthesis and test of LDR/PCR oligonucleotides using known DNA samples.** Oligonucleotides will be synthesized to recognize exons in SOD (on chromosome 21q), G6PD (on chromosome Xq), p53 tumor suppressor gene (on chromosome 17p), HER-2/neu (on chromosome 17q), and Int-2 (on chromosome 11q). These oligonucleotide primers have been designed so their ligation products have unique internal sequences, but the overall length and G+C content are identical (see Fig. 16). To minimize differences in ligation rates, the exon specific region of each primer set was chosen to ligate the junction sequence of (A,T)C↓C(A,T). This junction sequence corresponds to either a proline residue (codon CCN) or on the noncoding strand opposite a tryptophan residue (TGG). This particular sequence was chosen since tryptophan and proline residues tend to be conserved, and less likely to be a site of polymorphism. In addition, each sequence contains a single *Hae*III or *Hin*P1I restriction site at slightly different positions. Adjacent to the internal gene specific sequences are "adjustment sequences" (white bars), which equalize product length at 96 bases and G+C content at 52%. The external sequences of all oligonucleotides will be the same and complimentary to a pair of "zip code" PCR primers that will amplify only ligated products and not human sequences. One of the zip code PCR primers will be fluorescently labeled, while the other will contain a biotin group.

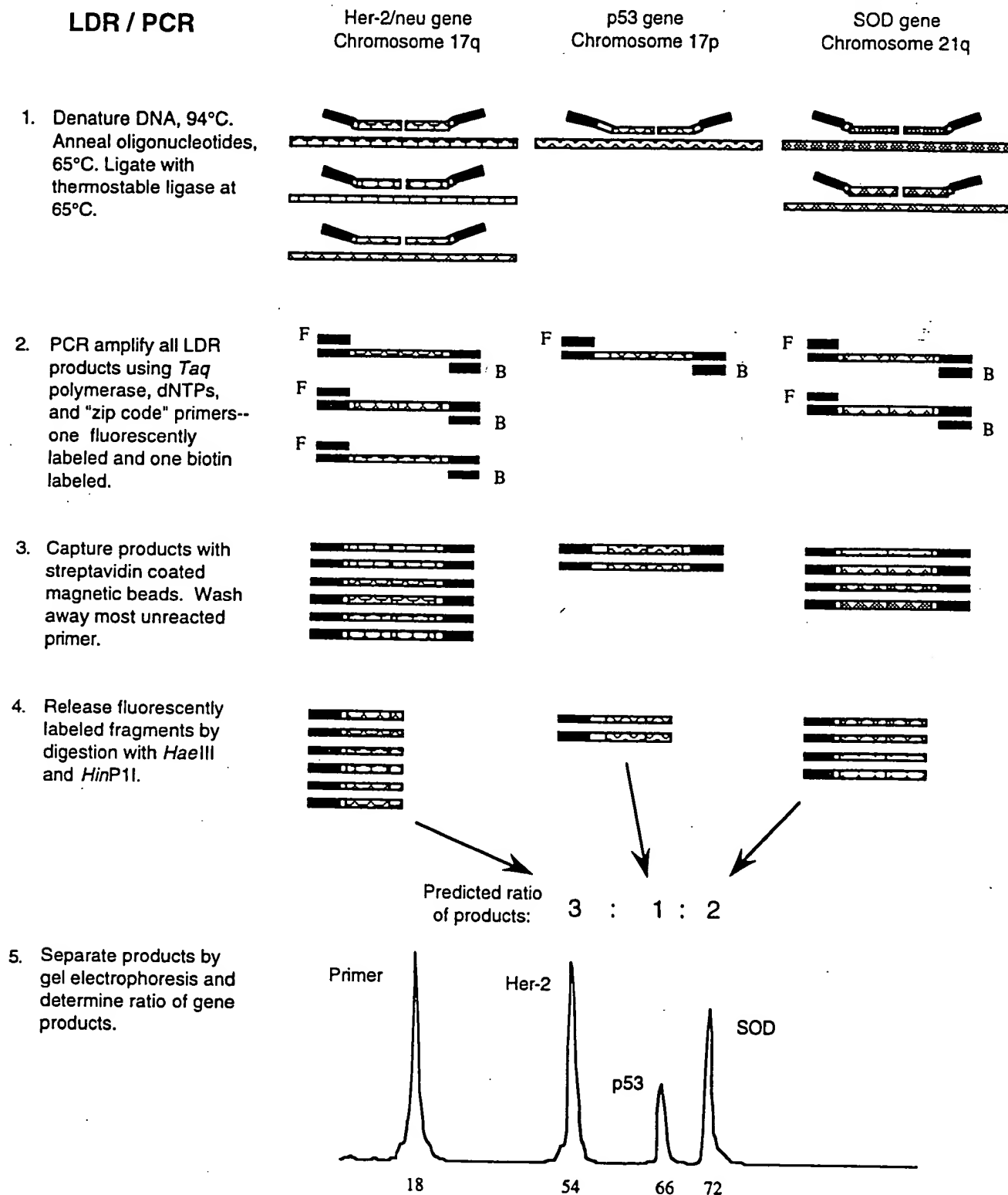


Fig. 15. Quantification of gene amplifications and deletions using the ligase detection reaction coupled to the polymerase chain reaction (LDR/PCR). Following denaturation, pairs of LDR primers anneal to their complementary templates and are ligated. Conditions will be optimized to obtain equal ligation efficiencies for all primer sets. Ligation with *Tth* ligase will be performed at 65°C well below the primers' designed  $T_m$  values of 75°C. All LDR products will be amplified simultaneously with *Taq* polymerase using two common "zip code" primers, thus maintaining proportionality. One zip code primer contains a fluorescent group, and the other a biotin group. Products may then be captured with streptavidin coated magnetic beads, washed, and digested with *HaeIII* and *HinP1I* to release fluorescently labeled fragments of unique size. These products may be separated on an ABI 373A DNA sequencer, and their ratio will be used to determine the relative copies of genes present in the initial target sample.

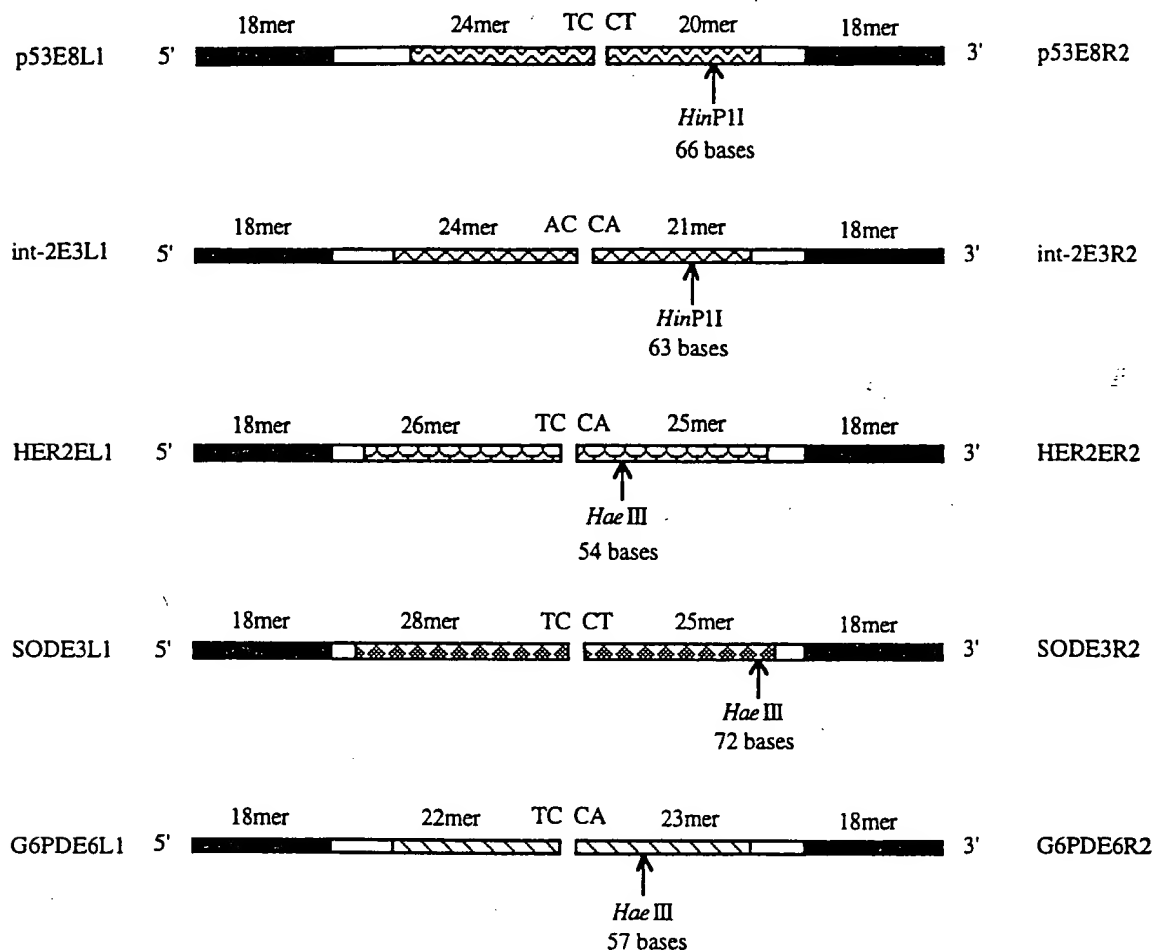


Fig. 16. LDR primers for quantification of gene amplifications and deletions. Oligonucleotides have been designed to recognize exon 8 in the p53 tumor suppressor gene (on chromosome 17p), exon 3 of int-2 (on chromosome 11q), an internal exon in HER-2/neu (on chromosome 17q), exon 3 in SOD (on chromosome 21q), and exon 6 in G6PD (on chromosome Xq). Each LDR primer pair has the following features: (i) The left primer contains from 5' to 3' an 18 base sequence identical to the fluorescently labeled zip code primer (black bar), an "adjustment sequence" (white bar), and a gene-specific sequence of from 22 to 28 bases with a  $T_m$  of 75°C (patterned bar). (ii) The right primer contains from 5' to 3' a gene-specific sequence of 20 to 25 bases with a  $T_m$  of 75°C (patterned bar), a single *HaeIII* or *HinfPII* restriction site at slightly different positions within the gene-specific sequence, an "adjustment sequence" (white bar), and an 18 base sequence complementary to the biotinylated zip code primer (black bar). (iii) The adjustment sequences (white bars) are designed such that the combined length of the two primers is exactly 96 bases, with 50 G+C bases and 46 A + T bases. The position of each unique restriction site generates a product which differs by at least 2 bases from the other products. (iv) The exon specific region of each primer set was chosen to ligate the junction sequence of (A,T)C↓C(A,T). This junction sequence corresponds to either a proline residue (codon CCN) or the complementary sequence of a tryptophan residue (TGG). These sequences were chosen to minimize differences in ligation rates and chance of a polymorphism at the ligation junction. Sequences of oligonucleotides are available upon request.

Ligation reactions will be carried out using test genomic DNA and the appropriate oligonucleotides (e.g. SOD and G6PD oligonucleotides for male, female, and trisomy 21 DNA's). Several ligation conditions will be tested to assure equal rates of ligation for each primer set. Ideally, ligations should go to completion, so initial ligations tested will be 10°C below the oligonucleotide  $T_m$  value. The amount of ligation product may be proportionally increased by a few additional LDR cycles (1 to 5 cycles), but not so many as to have product inhibition of the ligation reaction. After ligation, *Taq* polymerase with the zip code primers will be added, and PCR amplification will be performed. (We are aware that one LDR oligonucleotide may also serve as a primer and the other as a template for polymerase extension. Although products from those reactions may hybridize to generate a substrate for further PCR amplification, such products will not form in the absence of genomic DNA, and will be an insignificant percentage of the authentic LDR/PCR product.)



Products will be captured with streptavidin coated magnetic beads, washed of unreacted fluorescent primers, and released by digestion with *Hae*III and *Hin*PII. This will yield fluorescently labeled fragments of different sizes which can be separated and quantified on an ABI 373A DNA sequencer. The ratios of products will be determined using the Genescan 672 software. Multiple reactions will allow us to calculate means and 95% confidence intervals. As controls, different ratios of individual PCR amplified products will be mixed and diluted for LDR/PCR. To test for equal PCR efficiencies among the LDR products, a solution will be prepared with the LDR products in known ratios. By PCR amplifying from this solution and quantifying the products at different cycles we will be able to see whether the product ratios remain the same. It may be necessary to take samples after fewer rounds of PCR amplification to avoid skewing of the product ratios as the PCR reaction plateaus.

The above protocol may be generalized to accommodate quantification of gene amplification or deletion at several dozen loci simultaneously, without the need for a conveniently placed restriction site. Following LDR ligation, the products are proportionally amplified using one unlabeled and one biotinylated zip code primer. Products will be captured with streptavidin coated magnetic beads, washed of unreacted primers, and the DNA made single stranded by washing the beads in base. Addition of a molar excess of fluorescent zip code primer and several gene specific adjacent primers (containing different size tails) which hybridize on the single stranded product allows for quantitative (90% or better) ligation in the presence of thermostable ligase. Unreacted primers are removed by washing, and the ligated products released in a small volume of base. The fluorescently labeled ligation products will be of different sizes which can be separated and quantified on an ABI 373A DNA sequencer. Alternatively, the gene specific adjacent primers will contain array specific zip codes, which will be captured by the appropriate oligonucleotide or PNA addressable array, and quantified on a Molecular Dynamics Fluorimager 575 (See Project 5 and Core B.)

The ability to simultaneously detect both small and large deletions has additional benefits for cancer research. By judiciously selecting genes from both the long and short arms of each chromosome, this LDR/PCR method could be used to generate a molecular karyotype. This could help identify additional loci associated with tumorigenesis. Once a broad chromosomal region is identified, the same LDR/PCR method could help develop a fine structure map of the region, analogous to the initial discovery of DCC [101].

In addition, small deletions or insertions of unpredictable size in tumor suppressor genes might be easily identified by synthesizing sets of LDR primers to cover every 6-10 bases in the coding region of the gene. (This will become more feasible as improvements in oligonucleotide synthesis instrumentation will allow for synthesis of dozens to hundreds of oligonucleotides at a time.) For example, in a literature survey, we identified 151 mutations in the APC gene of which 15 were insertions (9.9%), 52 were deletions (34.4%) and 84 were single base changes. All 67 insertions and deletions (44.3%) could be easily identified by using just 25 sets of LDR primers, and one set of zip code primers. The remaining single base changes could be identified by the standard PCR/LDR methods described in specific aim (i) above.

(b) *Sensitivity of quantification in mixed tumor/normal cell populations.* The detection of X chromosome gene dosage in male and female genomic DNA's is a model for the deletion of a single allele in cancer cells. The additional chromosome in trisomy 21 genomic DNA mimics a 50 percent gene amplification. The sensitivity of our technology can be determined by examining these model situations and by mixing male and female and trisomic and normal cells. Quantification of LDR/PCR products from multiple reaction tubes will allow us to determine gene dosage with 95% confidence limits based on the *t* test. We will need to determine the number of observations (samples) required to achieve the appropriate confidence limits for detecting a single allele deletion or 50 percent gene amplification in half of the cells in a tumor. Initial experiments will use male, female, normal, and trisomic cells as well as mixtures of these DNA's (e.g. trisomic DNA diluted with normal DNA) to test a wide range of possible gene ratios. These studies will be extended to detect gene amplifications and deletions in previously characterized breast tumor cell lines. Dilution of cultured breast tumor cells with normal cells will also help us establish the limits of the LDR/PCR method.

(c) *Detection of gene amplification or deletion in frozen and fixed tissue.* Our sample set of 100 to 200 breast tumors will be investigated for deletion of p53, and amplification of HER-2/neu and/or int-2, compared to control genes SOD and G6PD by simultaneously amplifying LDR products from all these sites. Fixed



tumor specimens corresponding to the frozen samples will be examined to develop the technology for use on formaldehyde-treated, paraffin-embedded specimens. Bone marrow aspirate samples corresponding to these primary tumors have been investigated for micrometastases by immunohistochemical techniques. Many of them have also been characterized for HER-2/neu amplification by southern blotting [125]. The results of our studies of HER-2/neu and int-2 amplification in this tumor set will be correlated with the previously explored laboratory parameters and clinical prognostic parameters (e.g. primary tumor size, lymph node status and estrogen receptor status). We recognize that our p53 LDR primers have their junction in exon 8 at codon 278, and thus may fail to ligate when used on tumors containing a mutation at or near this codon. Such a result would be falsely interpreted as a p53 deletion instead of just a single base mutation. However, the interpretation of this data, namely, that the sample had lost a *functional* p53 gene remains the same.

**(iii) Development of a polymerase chain reaction/restriction endonuclease/ligase detection reaction (PCR/RE/LDR) to detect and identify mutations at a sensitivity of 1 in  $10^6$  or  $10^7$  cells.** A general method to detect any rare cancer cell carrying a mutation in any gene has been developed. The principle of this method is to continuously remove normal sequences while selectively amplifying the cancer mutation. A two base region of interest is amplified using PCR primers creating a restriction endonuclease recognition site. Treatment with the cognate endonuclease selectively cleaves product arising from the wild-type sequence. The cancer mutation resists cleavage, and thus is selectively amplified. Use of LDR or LCR primers distinguishes the authentic mutation from background primer dimer or polymerase misincorporation products during the detection step. This method will be expanded to demonstrate conversion of *any* dinucleotide (16 possibilities) into one of nine special endonuclease recognition sites. Use of multi-pairing or universal spacer nucleotide analogues will enhance significantly the efficiency and specificity of several of these site conversions (See Project 3). The p53 gene codon 248 has been converted to a *TaqI* site, demonstrating the feasibility of this approach. We plan to extend the technology to include conversion of breast cancer "hot spot" codons V157, R175, R273, and R282 into *TaqI* sites. Should primary breast tumors contain mutations in these 5 hot spots, lymph nodes and bone marrow samples from the same patients will be tested by PCR/RE/LDR for the presence of tumor cells. In this initial study, the presence of micro-metastases, will be correlated with HER-2/neu and int-2 gene amplification. Ultimately, PCR/RE/LDR on blood samples might become a powerful tool for detecting early metastasis or relapse.

*(a) Overview of generalized amplification of any dinucleotide sequence.* The principles already demonstrated for converting a CG dinucleotide into a *TaqI* site are generally applicable to converting any dinucleotide pair into an appropriate restriction site. The use of such conversions in a cancer detection assay with a one in  $10^6$  fold sensitivity depends on the following three properties of the thermostable polymerase.

Thermostable polymerase extension of the mismatched primer must:

- (i) *be significantly more efficient than extension at an incorrect position or formation of primer dimers;*
- (ii) *proceed with high fidelity for the very next nucleotide added;*
- (iii) *convert essentially 100% of the wild type sequence into the desired restriction site.*

Our model system used primers with a 3' T base to convert an *MspI* site (CCGG) into a *TaqI* site (TCGA). This was an ideal site for conversion, since *Taq* polymerase efficiently extends a T:G, T:C, or T:T mismatch, especially at high dNTP concentrations [71, 90]. However, high dNTP concentrations also favor formation of primer dimers and may decrease *Taq* polymerase fidelity [87, 88].

As part of our program project, we have devised schemes to overcome potential difficulties in converting any dinucleotide pair into the appropriate restriction site at high fidelity. These schemes include: (i) *Synthesis of primers containing base analogues designed to help convert one base pair into another preferred base pair (See Project 3);* (ii) *Development of assays to test the fidelity and efficiency of extending normal and analogue containing primers (See Core B);* and (iii) *Exploring use of different polymerases with and without proofreading activity (See below).* (iv) *Testing of restriction endonucleases using different primer configurations (See below).*

*(i) Synthesis of primers containing base analogues designed to help convert one base pair into another preferred base pair.* A new class of nucleotide analogues, herein termed "convertides" have been designed to

convert a wild type sequence into one which contains a restriction endonuclease recognition site. (Synthesis of convertides designated Q<sub>1</sub>-Q<sub>17</sub> will be covered in detail in Project 3.) A successful convertide will pair to one or more natural bases in a hybridization reaction allowing for efficient extension by *Taq* polymerase ("read step"), and also function as a template for incorporation of another base as the *Taq* polymerase copies the primer containing strand ("write step"). As an example, consider the problem of converting a T:A base pair into a G:C base pair. By adding 10% primer containing inosine ("Q<sub>10</sub>" see Project 3) as the 3' base, the amplification can be "jump started" as *Taq* polymerase will extend an I:A mismatch more efficiently than a G:A mismatch. On the second round of polymerase extension, the C nucleotides will be preferentially inserted opposite the inosine base in the primer. Thus, insertion of the C base will allow the remaining 90% primer (ending in G) to extend efficiently. Once the process has initiated, it is self sustaining. Primers containing the nucleotide analogue will contain three base mismatched "tails" on their 5' end. This will avoid extension of nucleotide analogue product in the second round of amplification, since such products may be resistant to restriction endonuclease digestion.

(ii) *Development of assays to test the fidelity and efficiency of extending normal and analogue containing primers.* The nucleotide analogues generated in Project 3 will be incorporated into tester oligonucleotides to access their ability in converting a given sequence into an endonuclease recognition site. We have devised a series of assays to answer how well a nucleotide analogue can "read" a natural base, and what bases the polymerase will "write" opposite the analogue. Those analogues which demonstrate versatility in both the read and write steps will be incorporated into a second set of oligonucleotides. These oligonucleotides are designed to test the efficiency and fidelity of polymerase extension for all 12 possible conversions. The assay is designed to mimic the type of zip code conversion required for cancer detection. These experiments are described in greater detail in Core B.

(iii) *Exploring use of different polymerases with and without proofreading activity.* A diagram of how convertides may be used for converting a sequence into a restriction endonuclease site is shown in Fig. 17A. *Taq* polymerase, which lacks a 3'→5' proofreading activity could extend the Q:G base pair with higher fidelity and efficiency than a T:G mismatched base pair. A series of assays has been developed to test the fidelity and efficiency of extending a given Q:natural base as described in Core B. If *Taq* polymerase fidelity of extending a mismatch proves problematic, an alternative approach would incorporate a thiophosphate group between the penultimate and the 3' end base. Such a thiophosphate group would allow use of a proofreading polymerases to increase the fidelity of extension as described in detail in section (c) below.

(iv) *Testing of restriction endonucleases using different primer configurations.* All 16 possible dinucleotide pairs can be converted into only **nine** restriction endonucleases recognition sequences (Table 2.) There are two types of candidate restriction endonucleases which are especially suited for this cancer detection method. The first type are thermophilic endonucleases which have four base recognition sequences. These are useful for designing primers analogous to those used with *TaqI* endonuclease. The second type contain interrupted palindromes, and preferably cleave with a 3' overhang. These allow one to essentially replace half of the recognition sequence. There are two approaches for designing primers for restriction endonucleases which recognize interrupted palindromes. (a) One can design one primer with mismatches to all nucleotides on one side as well as with one base on the other side. This primer is used in conjunction with a set of nested PCR primers which do not overlap the recognition sequence. (b) One can design one primer with mismatches to all nucleotides on one side, and the second primer mismatches the outside nucleotide on the second side. It must be experimentally determined which method is the more versatile and/or accurate one. Fig. 17B. below shows how either one or two mismatched primers may be used for conversion of a dinucleotide into a restriction site.

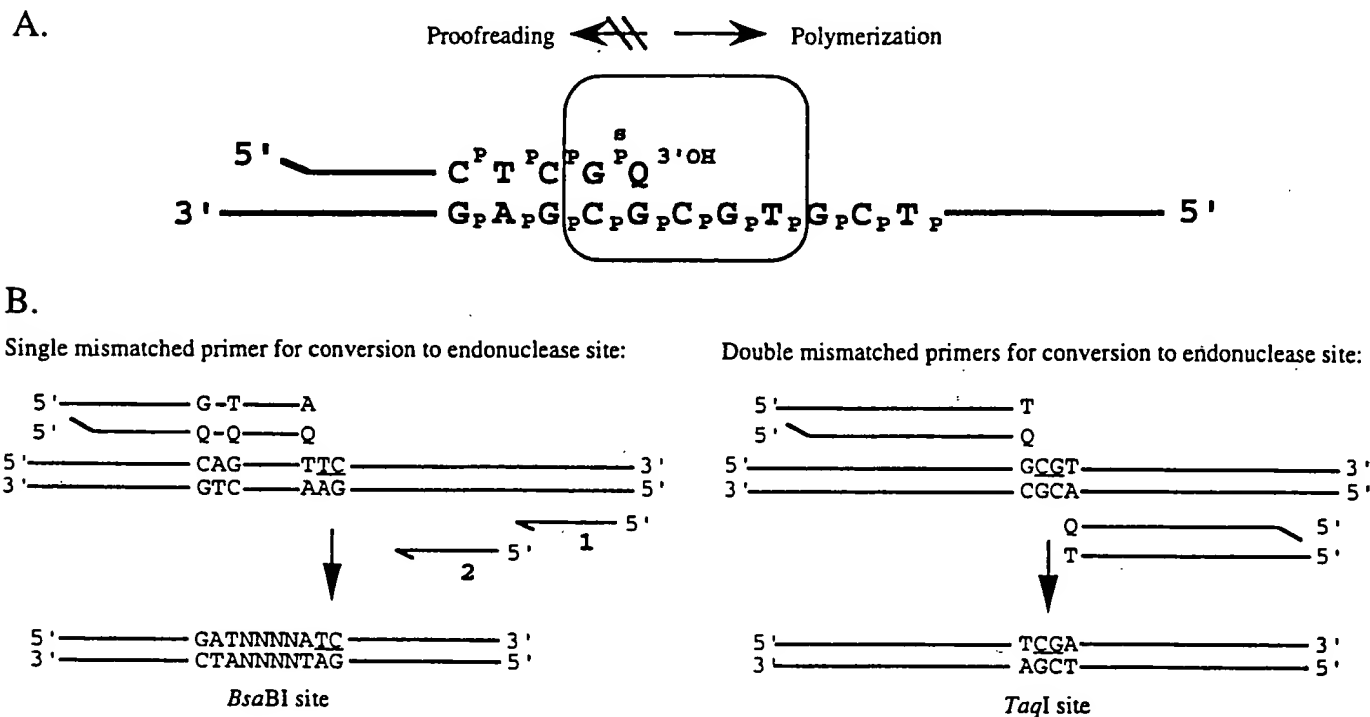


Fig. 17. Overview of parameters required for converting a normal DNA sequence into a restriction site. High sensitivity detection is at the underlined bases. Cancer mutations at these two positions will amplify and be resistant to restriction endonuclease digestion after conversion. **A.** Schematic diagram of polymerase with extension and proofreading activities. Thermostable polymerase is depicted as a gray rectangle binding to the 3' end of a primer DNA duplex. The primer contains a nucleotide analogue convertide (Q) at its 3' end to facilitate extension of a mismatch. *Taq* polymerase, which lacks 3'→5' proofreading activity can efficiently extend the Q:G base pair. Proofreading thermostable polymerase from either *Thermatoga maritima* [126], *Thermococcus litoralis* [127], or *Pyrococcus species GB-D* may exhibit even greater fidelity in adding the next base to the primer 3' end. However, these polymerases may also remove the 3' convertide base by the same 3'→5' proofreading activity. Removal of the convertide (Q) base may be avoided by incorporating a thiophosphate group between the convertide and the penultimate base. **B.** One sided and two sided mismatched primer conversion showing value of nucleotide analogues. **Left.** One sided mismatched primer conversion of a target sequence into a *BsaBI* site. The target DNA contains the sequence CAGNNNNNTTC. High sensitivity detection of a mutation in the underlined TC dinucleotide will require conversion of three bases in this sequences to form the *BsaBI* site GATNNNNATC. (Bases which need conversion are shown in **bold**). In the first PCR amplification (3 to 5 cycles), primer 1 and the convertide containing primer (just above the target DNA) are used. The convertides will allow the primers to hybridize to the correct sequence by pairing to the bases that need to be changed. During the first PCR amplification, *Taq* polymerase will insert other bases opposite the Q convertide, some fraction of which will be the desired bases. A second PCR amplification uses primer 2 and the upper top strand primer. This top primer will extend very efficiently on target containing perfectly matched DNA, and somewhat inefficiently on targets still containing one or two mismatches. Once the process has "jump started" the target will eventually be fully converted to a *BsaBI* site. Using nested primers 1 and 2 gives an added degree of specificity during the conversion process. **Right.** Two sided mismatched primer conversion of the GCGT target sequence into one containing a *TaqI* site (TCGA). In the first PCR amplification (3 to 5 cycles), both convertide containing primers (just above and below the target DNA) are used. The convertides will allow the primers to hybridize to the correct sequence by pairing to the bases that need to be changed. During the first PCR amplification, *Taq* polymerase will insert other bases opposite the Q convertides, some fraction of which will be the desired A bases. A second PCR amplification uses the extreme top and extreme bottom primers containing a T base on their 3' ends. This top primer will extend very efficiently on target containing perfectly matched DNA, and somewhat inefficiently on target still containing mismatches. Since these primers flank both sides of the two base sequence being analyzed, the advantage of using nested primers is lost. The problem is overcome by using primers containing zip code sequences (see Fig. 10) which would subsequently be used to amplify this fragment after restriction with *TaqI*. Non-homology between the last three bases of the convertide primers (depicted as a bent tail) and the authentic sequence primers assures that the convertide containing sequences will not be co-amplified.

Table 2. List of two base sequences which may be converted to restriction endonuclease recognition sites for detection of rare single base mutations.

Endo-nuclease	Recognition Sequence	Host Organism	Optimum Temperature	One mismatched Primer (a)		Two mismatched Primers (b)	
				3' base of conversion primer	Two base sequence detected	3' base of conversion primer(s)	Two base sequence detected
<i>TaqI</i> *	T↓CGA	<i>T. aquaticus</i>	65°C			T	CG #
<i>TthHB8I</i> *	T↓CGA	<i>T. thermophilus</i>	65°C			T	CG
<i>MwoI</i> *	GCNNNN↓NNGC	<i>M. wolfeii</i>	55°C	N	GC #		
<i>BstUI</i> *	C↓GCG	<i>B. stearotherm.</i>	60°C			C	GC
<i>TmaI</i>	C↓GCG	<i>T. maritima</i>	95°C			C	GC
<i>Tsp509I</i> *	↓AATT	<i>T. species</i>	65°C			A	AT
<i>TruII</i>	GATC	<i>T. ruber</i>	55°C			G	AT
<i>Bst1274I</i>	GATC	<i>B. stearotherm.</i>	60°C			G	AT
<i>MseI</i> *	T↓TAA	<i>M. species</i>	37°C			T	TA @
<i>BsaBI</i> *	GATNN↓NNATC	<i>B. stearotherm.</i>	60°C	A	TC, GA #	G	AT, AT #
<i>Tth111I</i> *	GACN↓NNGTC	<i>T. thermophilus</i>	65°C	G	TC, GA	G	GT, AC #
<i>TspI</i>	GACN↓NNGTC	<i>T. species</i>	65°C	G	TC, GA	G	GT, AC
<i>BsII</i> *	CCNNNN↓NNGG	<i>B. species</i>	55°C	N	CC, GG #	G	C, G
<i>BstXI</i> *	CCANNNN↓NTGG	<i>B. stearotherm.</i>	55°C	T	CC, GG	C	TG, CA #
<i>BstWI</i>	CCTNNNNNAGG	<i>B. stearotherm.</i>	55°C	A	CC, GG	C	TG, CA
<i>DraIII</i> *	CACNNN↓GTG	<i>D. radiophilus</i>	37°C	G	TG, CA	C	GT, AC
<i>AlwNI</i> *	CAGNNN↓CTG	<i>A. lwoffii</i>	37°C	C	TG, CA	C	CT, AG @
<i>XmaI</i> *	GAANN↓NNTTC	<i>X. Manihotis</i>	37°C	T	TC, GA	G	TT, AA @

Enzymes are listed in groups according to dinucleotide sequences which could be converted to that recognition sequence. Some sequences may be easier to convert than others. (a) Some two base sequences may be converted into interrupted palindrome recognition sites using one primer with both an internal as well as a 3' base nucleotide conversion, and the other primer distal to the site. This allows for use of nested primers from the distal site. (b) For conversion of dinucleotides into contiguous four base recognition sites, both primers contain the 3' conversion base. When converting to an interrupted palindrome recognition site, only one primer contains a 3' conversion base, while the other contains internal conversion bases. Asterisk (\*) indicates enzyme is commercially available. Number (#) indicates these "top six endonucleases" are already thermophilic. The vast majority (94%) of point mutations reported in the p53 tumor suppressor gene should be detectable at 1 mutation in  $10^6$  using these top six endonucleases. The remaining three mesophilic enzymes (indicated with at sign, @) would detect the remaining 6%.

Besides *TaqI*, the most valuable thermophilic endonucleases are *BsII* and *MwoI*, which have four base recognition sequences interrupted by seven base pairs. These endonucleases require only one primer which contain only two internal mismatches seven bases from the 3' end. Since the second primer is not needed to create a *BsII* or *MwoI* site, a series of three nested primers may be used in successive rounds of amplification. As we already demonstrated with our PCR/*MspI*/LCR experiments (see preliminary results and Project 1), use of nested primers has the advantage of avoiding reamplification of primer dimer products. Thus, conversion of CC, GG, and GC to *BsII* and *MwoI* respectively, should give exceedingly sensitive detection of rare mutations using the procedures described in preliminary results section (iii b). These three endonucleases, *TaqI*, *BsII*, and *MwoI*, could be used for selectively amplifying 212 of the 326 point mutations (=65%) reported for the p53 tumor suppressor gene [52]. The endonucleases which could be used for selectively amplifying the other 35% of point mutations in the p53 gene are shown in Fig. 18.

		2nd Base			
		T	C	G	A
1st Base	T	TT 1. 2. <i>XmnI</i> 3	TC 1. <i>BsaBI</i> , <i>Tth111I</i> 2. 5	TG 1. <i>DraIII</i> , <i>AlwNI</i> 2. <i>BstXI</i> 12	TA 1. 2. <i>MseI</i> 1
	C	CT 1. 2. <i>AlwNI</i> 7	CC 1. <i>BstI</i> , <i>BstXI</i> 2. 17	<u>CG</u> 1. 2. <i>TaqI</i> 130	CA 1. <i>DraIII</i> , <i>AlwNI</i> 2. <i>BstXI</i> 13
	G	GT 1. 2. <i>Tth111I</i> , <i>DraIII</i> 16	GC 1. <i>MwoI</i> 2. <i>TmaI</i> , <i>BstUI</i> 24	GG 1. <i>BstI</i> , <i>BstXI</i> 2. 41	GA 1. <i>BsaBI</i> , <i>Tth111I</i> 2. 22
	A	AT 1. 2. <i>BsaBI</i> , <i>Tsp509I</i> 13	AC 1. 2. <i>Tth111I</i> , <i>DraIII</i> 14	AG 1. 2. <i>AlwNI</i> 3	AA 1. 2. <i>XmnI</i> 5

Fig 18. Summary of restriction endonucleases which could be used to selectively amplify mutations in the p53 tumor suppressor gene. A recent review listed 326 point mutations in the p53 tumor suppressor gene [52]. The DNA sequence in any gene can be written as a combination of the sixteen dinucleotide sequences. Therefore any mutation in that gene will disrupt a dinucleotide sequence. This figure lists all the 16 dinucleotide pairs as a 4 x 4 matrix. Within each cell of this matrix is listed from top to bottom: the dinucleotide sequence, restriction endonuclease(s) which may be used for one (1.) sided conversion of any sequence to that site, restriction endonuclease(s) which may be used for two (2.) sided conversion of any sequence to that site, and the number of mutations in p53 (out of 326) which could be detected by this conversion. When more than one endonuclease is listed, the preferred enzyme is underlined. The most commonly required conversion is of a CG dinucleotide into a *TaqI* site. This reflects the CG dinucleotide hot spots in the p53 gene at codons 157, 175, 248, 273, and 282 [52]. Note that the entire matrix may be covered by only nine restriction endonucleases: *TaqI*, *BstI*, *MwoI*, *BsaBI*, *BstXI*, *Tth111I*, *AlwNI*, *XmnI*, and *MseI*.

Six of the nine endonucleases required for detecting point mutations are already thermophilic, and can be used to detect over 90% of point mutations reported in the p53, Ha-ras, Ki-ras, N-ras, and APC genes. Based on our proposal, Dr. Richard Roberts, Dr. Ira Schildkraut, and Dr. Geoffrey Wilson of New England Biolabs Inc. will expand their restriction endonuclease screening program to search for additional thermophilic enzymes. This will include testing about 40 *B. stearothermophilus* and *Thermus* species strains collected by the P.I. during the summer of 1991 in Yellowstone [128, 129].

We are aware of the advantages of using a restriction endonuclease which is both *thermophilic* as well as *thermostable*. To be effective in an amplification reaction, a *thermostable* enzyme must not become irreversibly denatured when subjected to the elevated temperatures (about 90°C - 100°C) for the time necessary to effect complete denaturation of double-stranded DNA (about 30 to 60 sec). Both *Taq* polymerase and *Taq* ligase retain activity after 30 or more repeated 1 minute- exposures to 94°C [53, 54, 130, 131], and hence are termed *thermostable*. Unfortunately, the *Taq I* restriction endonuclease, isolated from the same thermophilic *Thermus aquaticus* species, does not survive such treatment (being completely inactivated after 20 min. at 85°C, [132]), and hence is only a *thermophilic* enzyme.

The *TaqI* endonuclease as well as the isoschizomeric *TthHB8I* endonuclease have been cloned, overproduced, sequenced, and biochemical properties characterized in the P.I.'s laboratory. [132-139]. We have now overproduced *TaqI*, *TthHB8I*, and two *TthHB8I-TaqI* chimeric hybrids by using gene assembly PCR (F. Barany unpublished results, and [140]). Preliminary experiments showed the following order of stability in MHS or 9LS buffer at 85°C: *TaqI* < *TthHB8I*<sup>1-93</sup>-*TaqI*<sup>94-263</sup> < *TthHB8I* < *TthHB8I*<sup>1-186</sup>-*TaqI*<sup>187-263</sup>. While *TaqI* was essentially inactivated after just 2.5 min. at 85°C, *TthHB8I* and *TthHB8I*<sup>1-186</sup>-*TaqI*<sup>187-263</sup> retained some activity even after 10 min. at 85°C. These two enzymes and additional thermostable enzymes we and collaborators construct or isolate will reduce or eliminate the need for three enzyme additions during the selection PCR step of our cancer detection work. Construction of a fully thermostable *TaqI* restriction endonuclease is part of the P.I.'s NIH RO1 grant renewal, and no funds are requested in this program project for such work. Should construction of a thermostable *TaqI* restriction endonuclease be successful, it would be used in our cancer detection work.

(b) *Optimization of detection and quantification of rare mutation in p53 codon 248 by conversion to a TaqI site.* Our preliminary experiments demonstrate an ability to detect 1 cancer mutation in 10<sup>5</sup> wild type sequences by converting p53 codon 248 to a *TaqI* site (see background, above). We will attempt to further optimize our conditions to achieve sensitivities of 1 in 10<sup>6</sup> or even 10<sup>7</sup>.

The sensitivity of our cancer detection assay is dependent on the fidelity of polymerase extension of the base adjacent to the 3' mismatch. Our earlier work with detection of mutations in Ha-*ras* codon 12 indicate that *Taq* polymerase can achieve fidelity rates of better than 1 in 10<sup>8</sup> for a given base [84-86]. However, the fidelity of extension off a mismatched base-pair are unknown. There are several factors which may influence both the fidelity and rate of polymerase extension off a mismatched base pair. These include the nature of the mismatch, the polymerase used, the reaction temperature, pH, salt, nucleotide, and primer concentrations. Unfortunately, factors which enhance extension of mismatched bases may also reduce polymerase fidelity or encourage formation of (unwanted) primer dimers. We have therefore developed an assay to test the effect of such changes on polymerase fidelity.

A flow chart and more detailed diagram of our quantitative cancer detection assay are shown in Figs. 21 & 22. In our original detection scheme, the ratio of LDR product signals was compared for different dilutions of the cancer sequence into the wild-type sequence after repeated selection with *TaqI* endonuclease. This has been modified slightly to include addition of a marker sequence prior to the *TaqI* selection step. The marker sequence contains a different mutation (G) at the same nucleotide position as the cancer mutation (T). (This sequence will be generated *in vitro* using overlap mutagenesis PCR [140].) Thus, each reaction will have an internal control, and will not be subject to individual variation in endonuclease efficiency. Two tubes will be used, one containing a 10<sup>3</sup> fold dilution (left side) and the second containing a 10<sup>6</sup> fold dilution (right side) of the marker target in the amplified DNA mixture. The tube containing the 1 in 10<sup>3</sup> dilution of marker (left side) will undergo a single round of conversion to a *TaqI* site followed by biochemical selection with *TaqI* endonuclease. The selected products will be detected using LDR primers specific to both marker and mutant. Products will be separated either on an ABI 373 DNA sequencer or a capillary electrophoresis unit, allowing for peaks to be analyzed and quantified. (LDR primers for detecting the marker and cancer mutation are shown in Fig. 23.) Likewise, the tube containing the 1 in 10<sup>6</sup> dilution of marker (right side) will undergo two rounds of conversion and selection followed by detection. These experiments are designed to detect the presence of mutations from 1 in 10<sup>2</sup> to 1 in 10<sup>7</sup> DNA molecules. For example, should the mutation be present at a concentration of 1 in 10<sup>3</sup>, one would expect an approximately equal ratio of mutant and marker peaks from the lower dilution (left side) assay. If the mutation were present at a concentration of 1 in 10<sup>7</sup>, one would expect a 10 fold higher concentration of marker to mutant. All our initial experiments will start with mixing appropriate concentrations of p53 exon 7 amplified wild type product and mutant product to simulate different concentrations of cancer mutations in the range of 1 in 10<sup>2</sup> to 1 in 10<sup>7</sup>. Appropriate dilutions of marker target will be added to these test samples. A side by side comparison of amplification conditions varying the reaction temperature, pH, salt, nucleotide, and primer concentrations will be performed. *Taq* polymerase fidelity will be determined by quantifying mutant product peak in the absence of mutant target DNA. As an additional control, results with *TaqI* conversion will be compared with the standard *MspI* selection at p53 codon 248, which does not require site conversion (please see preliminary results section (iia) and Project 1).

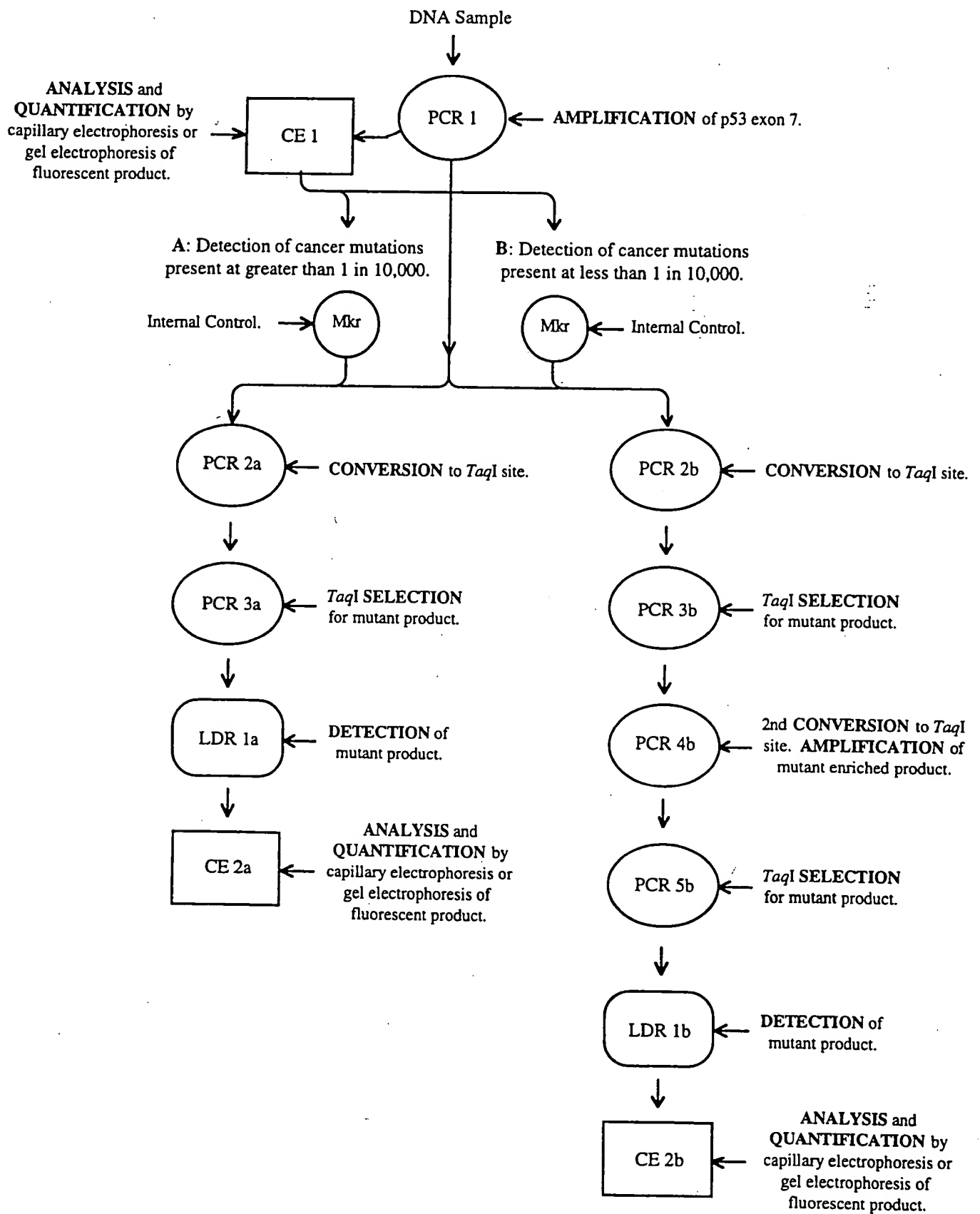


Fig. 21. Flow chart for quantitative PCR/RE/LDR cancer detection method. Please see Fig. 11 and 22.

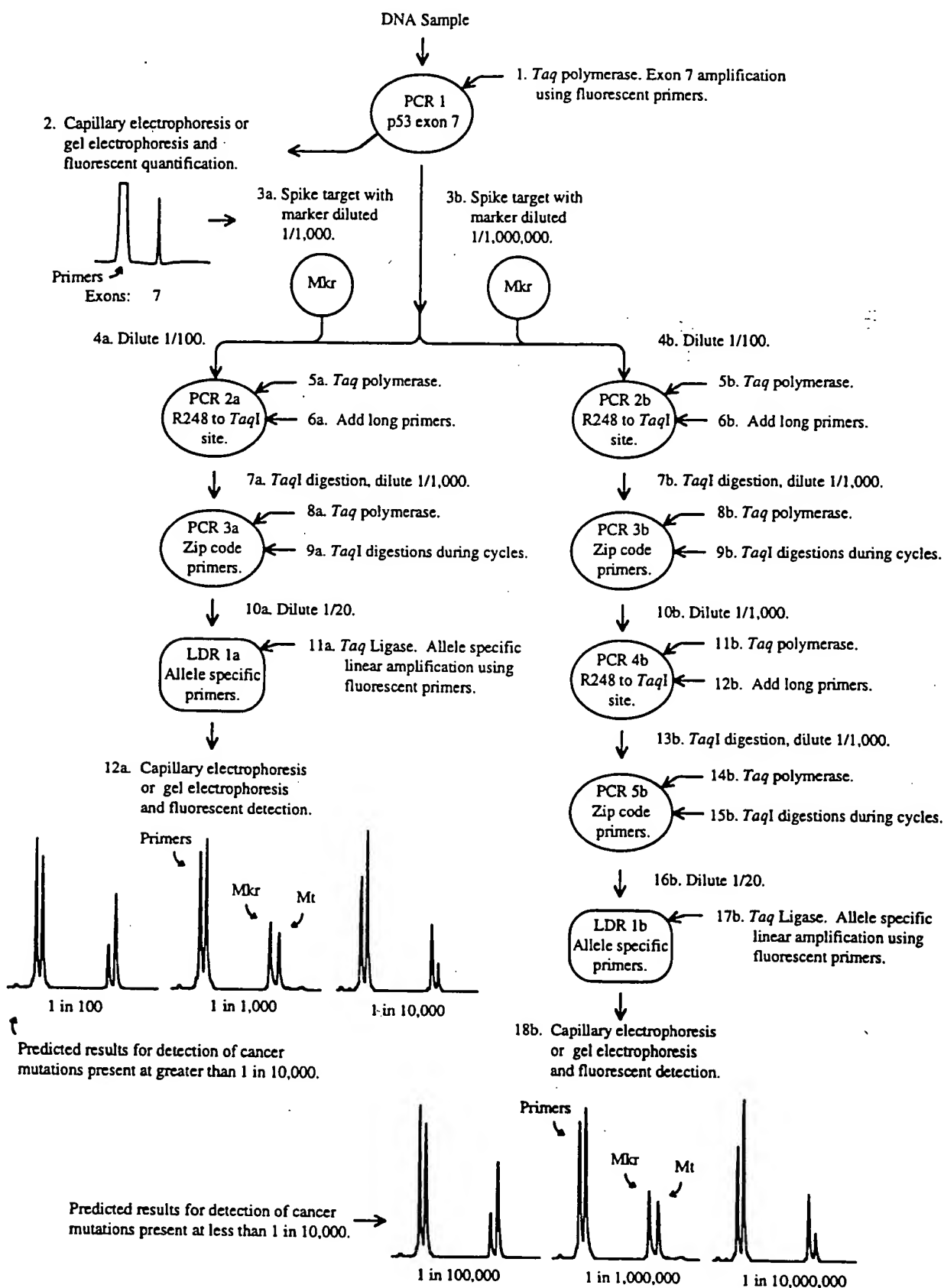


Fig. 22. Detailed flow chart for quantitative PCR/RE/LDR detection of from one mutation in  $10^2$  to one in  $10^7$  normal cells. Exogenous marker allows for accurate quantification. Chromatograms show predicted results for detection of cancer mutations present at greater than and less than  $1$  in  $10^4$ . Please see Fig. 12, 21 and 23.



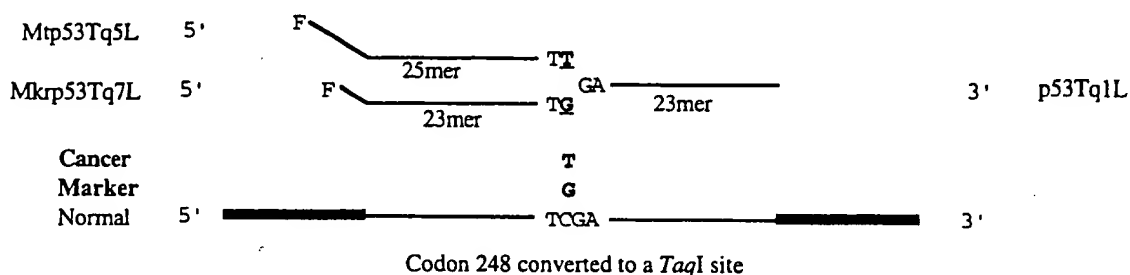
**Quantification of PCR/RE/LDR reaction by using a marker:**

Fig. 23. LDR primers for quantification of mutant product in a PCR/RE/LDR cancer detection scheme. Primers Mkrp53Tq3L and p53Tq1L are complementary to the p53 exon 7 sequence, except for the sequence TGGGA at codon 248. Thus these LDR primers will ligate only to an added marker target which has been converted to a *TaqI*-like sequence (TGGGA) at codon 248. This "marker mutation" R248G, has not been observed in codon 248 mutations to date, thus its presence identifies the marker sequence. Use of an added marker introduces a positive control, eliminating the uncertainties of using wild type DNA as an internal control. Primer Mtp53Tq5L contains a T on its 3' end. Thus, it will ligate to p53Tq1L only if mutant target has been converted to a TTGA sequence at codon 248. This would correspond to detecting an R248W mutation at codon 248. Primers Mkrp53Tq3L and Mtp53Tq5L are fluorescently labeled and of different lengths so their ligation products may be distinguished and quantified on an ABI 373A DNA sequencing apparatus.

These experiments will determine if we can improve our detection of a mutation in codon 248 from 1 in  $10^5$  to 1 in  $10^7$  or better. The results will suggest either further improvements of the technology (see overview, above), or allow us to expand our assays to clinical samples.

(c) *Further optimization of detection and quantification of rare mutation in p53 codon 248 by conversion to a *TaqI* site.* Based on our results from section (b) above, we will determine if additional improvements in the fidelity of polymerase extension are required. These two complementary approaches are: (i) Use of nucleotide analogues in conversion primers, and (ii) Use of a thermostable polymerase with proofreading activity.

(i) *Use of nucleotide analogues in conversion primers.* As an initial approach, we will synthesize the short conversion oligonucleotides p53Tq248C and p53Tq248D with nucleotide analogues as the 3' base instead of the T (see Fig. in preliminary results, and Fig. above). These nucleotide analogues should mimic T yet base pair to G. The first two analogues we will test are Q<sub>11</sub>, and Q<sub>12</sub>, both of which have been reported in the literature (see Project 3). These oligonucleotides will also contain a 3 base mismatch at their 5' end to avoid incorporation of an incorrect base at the conversion site into the larger product (see Fig. section above). We will compare the efficiency of site conversion as well as the fidelity of extension between the nucleotide analogue containing primers and the original primers (ending in 3'T) using the marker/mutant assay describe in section b above. Should these results suggest that nucleotide analogues give a substantial improvement over the T:G mismatch, they would be incorporated into future assays. We are also aware that different polymerases may have different extension and fidelity profiles for a given nucleotide analogue/natural base pair. Therefore, we would compare results with non-proofreading polymerases from *Thermus aquaticus* "Taq" [130], *Thermus thermophilus* "Tth" [141], and *Thermococcus litoralis* exo3-[142]. In addition, we would also compare primers ending in the best nucleotide analogue with primers containing a 3' Q<sub>3</sub>, or Q<sub>4</sub> analogue. Note that these analogues may not show substantial improvement over a T:C mismatch, but may show improvement over a T:C mismatch. A general assay to test polymerase efficiency of site conversion and fidelity of extension for effecting all 12 possible conversions is presented in Core B.

(ii) *Use of a thermostable polymerase with proofreading activity.* A second and complementary approach to increasing fidelity is to use a polymerase with 3'→5' proofreading activity. An important caveat is to insure that the proofreading activity does not remove the very mismatched base or nucleotide analogue one is trying to insert. This problem may be overcome by synthesizing an oligonucleotide containing a thiophosphate bond adjacent to the 3' base or nucleotide analogue [143]. A thiophosphate group is resistant to

exonucleolytic degradation [144, 145]. With this approach, both shorter and longer (zip code containing) primers would require a thiophosphate group adjacent to the 3' base. Experiments would be performed as described above using a proofreading thermostable polymerase from either *Thermatoga maritima* [126], *Thermococcus litoralis* [127], or *Pyrococcus species GB-D* [142]. (Please see letters of collaboration from Dr. David Gelfand, Roche Molecular Systems, and Drs. Richard Roberts, Ira Schildkraut and Geoffrey Wilson of New England Biolabs Inc.). Results with the proofreading polymerases ("*Tma*", "*Vent*", and "*Deep Vent*") would be compared to the non-proofreading polymerases ("*Taq*", "*Tth*", "*Vent exo3-*").

(d) *Caveats in achieving detection of mutations at a sensitivity of 1 in  $10^6$  or  $10^7$ .* A selection system which exponentially amplifies one signal while selectively removing another signal may be susceptible to inadvertently amplifying an incorrect signal. We have already encountered and solved some of these potential problems. They include: (i) Polymerase fidelity of extension off primers containing convertides or mismatched base pairs. (ii) Effect of truncated or thiophosphate oligonucleotide on conversion of sequence to a restriction site, and (iii) Presence of natural mutations in biopsy or tissue samples.

(i) *Polymerase fidelity of extension off primers containing convertides or mismatched base pairs.* In our initial studies using PCR/RE/LCR on the Ha-ras gene, we demonstrated detection of 10 mutated copies in  $10^9$  wild-type sequences [84-86]. The reported fidelity of *Taq* polymerase suggested that misincorporation of bases would limit this PCR/LCR procedures' sensitivity to less than one in  $10^6$  [87, 88]. However, the fidelity of *Taq* polymerase was determined as a misincorporation in any base within a large amplified region and the fidelity actually increases for any specific individual base. Fidelities of some proofreading polymerases are better than for *Taq* polymerase [126]. Nevertheless, it is important to include controls and determine the fidelity of polymerase extension, especially off primers containing convertides or mismatched base pairs, for each conversion under development. These assays are described in greater detail in Core B.

(ii) *Effect of truncated or thiophosphate oligonucleotide on conversion of sequence to a restriction site.* An important issue regarding primer synthesis needs to be addressed. While the presence of 1% truncated or modified nucleotide primer is of no consequence in an ordinary PCR reaction, it could have major effects when attempting to convert a given sequence into a restriction site. For example, if 1% of a thiophosphate containing primer had all normal non-bridging oxygens on the phosphate bonds, it would be susceptible to a polymerase 3'→5' exonuclease activity. The solution to this potential problem is two fold. First, one treats the thiophosphate primers with exonuclease III, which will degrade natural oligonucleotides 3'→5', but not thiophosphate containing oligonucleotides [144, 145]. The resistant primer is gel purified. Thus, a biochemical step is used to assure the primer will be "100%" pure. The second solution is to repeat the restriction site conversion step with a second round of PCR amplification. This has already proved successful in our original *TaqI* conversion of codon 248 (see preliminary results section iiib).

An added complication of using a thiophosphate oligonucleotide derivative is the possibility that this group may interfere with endonuclease cleavage. We have demonstrated that *TaqI* cleavage of a TCGA sequence containing a thiophosphate group 5' to the T is not inhibited, independent whether the thiophosphate group is in the "R" (pointing into the major groove) or "S" (pointing away from the major groove) configuration (A. Mayer and F. Barany, unpublished results). This is the position of the thiophosphate group in our assay. However, when the thiophosphate group is between the T and C (scissile bond) there is considerable inhibition (50 fold for the "R" isomer) or no detectable cleavage for the "S" isomer [139]. Thus, should a particular conversion require placement of a thiophosphate group at the scissile bond (for example, converting a CT dinucleotide to an *AlwNI* site), it would be necessary to remove the thiophosphate containing strands. This may be easily achieved by using biotinylated thiophosphate primers. After the site conversion, the DNA is diluted a thousand fold and reamplified using the zip code primers. The original biotinylated primers, now constitute less than 0.1% of the final product and may be removed by capture with streptavidin coated magnetic beads. The P.I.'s laboratory has considerable experience with this technology.

(iii) *Presence of natural mutations in the biopsy or tissue sample.* One of the important unanswered question in cancer detection is what is the background level of mutation at a given base in a human patient. Does an elderly patient have a higher background level? These questions will be addressed by analysis of normal tissue or blood from the patients with primary tumors, as well as control patients with no known tumors. In collaboration with Dr. Lawrence Grossman at Wayne State University School of Medicine, we

will try to determine the frequencies of mutations in mitochondrial DNA as a function of age and other factors. Parallel experiments will compare these frequencies to mutations in codon 248 of the p53 tumor suppressor gene.

The above series of experiments would test the efficiency of site conversion and the fidelity of polymerase extension using a standard assay. By use of nucleotide analogues, and/or proofreading polymerases with thiophosphate primers, we aim to significantly improve these values. A generalized assay to test the efficiency and polymerase fidelity of all 12 possible nucleotide conversions is presented in Core B.

(e) *Detection of rare cancer cells by demonstrating cancer-related mutations in clinical samples.* Primary breast tumor samples are available to us from 100 to 200 patients. The bone marrow specimens from many of these patients have been shown to contain micrometastatic lesions by immunohistochemical staining [125]. In part (i) of this methods section we describe how we will investigate these primary breast tumor samples for any of 24-40 mutations. Five p53 mutations (V157, R175, R248, R273, and R282) occur in about 23 percent of breast cancers. Fortuitously, four of these codons occur at naturally occurring restriction sites; *MspI* at V157, R248, and R282, and *HhaI* at R175. Codon R273 is only one conversion away from a *BstUI* site. All five of these codons can also be converted to *Taq I* sites by PCR/RE/LDR. (See above.) We will attempt to detect micrometastases of breast cancer cells to lymph node and bone marrow specimens from the patients whose primary tumors contained any of these five mutations.

We will identify the p53 mutations on the 100 to 200 breast tumor samples using PCR/LDR. Lymph node tissue and bone marrow aspirate slides will be obtained on those tumors with mutations at V157, R175, R248, R273, and R282. DNA will be extracted from lymph node specimens and bone marrow aspirates. Exons 5, 7 and 8 of p53 will be amplified. The mutation found in the primary tumor will be sought from the corresponding lymph nodes and bone marrow specimens using PCR/RE/LDR. Experiments will be performed in parallel, comparing results obtained using (i) Zero conversion primers, with both sides nested, such as *MspI* at R248, (ii) One conversion primer and one side nested, such as *BstUI* at R273, and (iii) Two conversion primers with neither side nested. Searching for an inappropriate mutation (e.g. searching for a V157 mutation in the bone marrow of a patient whose primary tumor contained an R248 mutation) will act as a negative control.

Results of our experiments will be correlated with the results of immunohistological screening for micrometastases. From 100 to 200 primary breast tumors we would expect to find about 23 to 46 with mutations at V157, R175, R248, R273, or R282. Of these we would expect four to eight to have micrometastases to the bone marrow by immunohistochemistry. A similar number would have micrometastases to lymph nodes.

Wide acceptance of PCR/RE/LDR will require detection of p53 mutations by conversion to restriction sites other than the *TaqI* endonuclease. If the above experiments and controls give promising results, the PCR/RE/LDR protocols will be expanded to include common p53 mutations at Y163 (2.7% of breast cancers; two primer conversion to a *Tth* 111 site), L194 and R280 (both at 2.7%; both two primer conversion to an *AlwNI* site), and M237 (2.7%; natural *NlaIII* site, or two primer conversion to a *BstXI* site). In collaboration with Project 1 we also plan to develop PCR/RE/LDR protocols to include p53 mutations at H179 (3.0% of lung cancers; natural *NlaIII* site, or one primer conversion to *BstXI* site), C242 (3.4% of lung cancers; one primer conversion to *MwoI* site), G245 (2.1% of lung cancers; one primer conversion to *BsII* site), and R249 (3.0% of lung cancers; natural *HaeIII* site, or one primer conversion to *BsII* site).

Although it is beyond the scope of project 2, we recognize that a highly sensitive method which detects one mutant cell in  $10^6$  or  $10^7$  normal cells may be useful for identifying cancer cells circulating in the blood stream (Please see Project 1). Large scale studies will be necessary to correlate mutations in primary tumors with the presence of circulating cancer cells, as well as the prognostic significance of cancer cells in the blood. Early relapses might be detected by identifying circulating cells having the same mutations found in the primary tumor. In the future the existence of primary tumors might be demonstrated by blood testing.

## E. PROGRAM ASPECTS

We are developing a new approach to multiplex detection of point mutations, gene amplifications and deletions in tissue samples associated with breast and cervical cancers. These approaches combine the sensitivity of PCR with the specificity of LDR. The three parts to this program are: (i) Development of a multiplex polymerase chain reaction/ligase detection reaction (PCR/LDR) system for detection of point mutations in tumor biopsies. This should eventually lead to rapid and simultaneous detection of 24 to 40 point mutations, representing from 63% to 79% of mutations, in the p53 gene from breast tumor specimens. The method will also be applied for simultaneous detection of high risk HPV in cervical lavages or biopsies. (ii) Development of a ligase detection reaction/polymerase chain reaction (LDR/PCR) system for detection of gene amplifications and deletions in tumor biopsies. This should allow for simultaneous detection of HER-2/neu and int-2 amplifications and deletion of p53 in breast tumor specimens. (iii) Development of a polymerase chain reaction/restriction endonuclease/ligase detection reaction (PCR/RE/LDR) to detect and identify mutations at a sensitivity of 1 in  $10^6$  or  $10^7$  cells. This high sensitivity will allow for detection of micrometastases to lymph nodes and bone marrow. The presence of micrometastases will be correlated with HER-2/neu and int-2 gene amplification. Ultimately, this high sensitivity method may help identify circulating cancer cells in the blood and thus detect early relapse or the presence of a primary tumor.

Projects 1 and 2 are parallel approaches to detecting cancer causing mutations, found in the lung and colon (Project 1), or the breast and cervix (Project 2). Advances in one project will immediately be communicated to work in the other. These two projects will build on the accomplishments provided by the design and synthesis of nucleotide analogues (Project 3) which serve not only as convertides, but also as universal base pairs. Basic research aimed at engineering an improved thermostable ligase (Project 4) may significantly increase the sensitivity of our PCR/LDR and other cancer detection methods. The ability to simultaneously detect hundreds of cancer mutations awaits the powerful new approaches to synthesizing addressable arrays (Project 5). Both the informatic support for cancer detection, and the instrumentation and mutation detection cores (Core A and Core B) play a critical role in achieving our goal of high sensitivity and specificity with multiplex cancer mutation detection.

We recognize that our narrow focus on the p53 gene is simply the first step toward ultimate goal of understanding the molecular mechanics of cancers. Although p53 has been implicated in about 50% of cancers, it still leaves an astonishingly high number of tumors where the primary genetic defect is unknown. Dr. Mark Sobel of the National Cancer Institute has independently used LCR to identify new mutations affecting homeodomain genes in breast cancer tissues [146], (see letter of collaboration). For mutations that Dr. Sobel and others discover, one must determine (i) whether the mutation is significant or simply a polymorphism (ii) whether the gene plays a role in a specific cancer or cancers in general, and (iii) whether the mutation may be used diagnostically or prognostically. The innovative method of difference cloning [57,58] can yield hundreds of mutations which are different between normal and tumor tissue [147]. This bewildering number of mutations which might lead to new genes that play a role in cancers urgently requires high throughput mutation detection methods to assess their significance.

The ability to simultaneously detect a variety of mutations at exquisitely high sensitivities will be of benefit to several of our collaborators. This will aid in detection and identification of: pathogenic microorganisms by identifying 16s polymorphisms (Dr. Carl Batt), multiple drug resistant strains of *Mycobacterium tuberculosis* in mixed infections (Dr. David Persing), dozens of  $\beta$ -lactamase mutations responsible for third generation  $\beta$ -lactam resistance (Dr. Patrice Courvalin), pathogenic wild type revertants which interfere with polio virus vaccine production (Dr. Olen Kew), mutations associated with ageing (Dr. Larry Grossman), polymorphisms in the E6 and E7 genes of high risk HPV strains (Dr. Saul Silverstein), multiple germline mutations in single gene disorders (Dr. Eric Hoffman, Dr. Perry White, and Dr. Emily Winn-Deen), and multiple somatic or germline mutations in tumor suppressor genes and oncogenes (Dr. Jack Fishman, Dr. John Kovach, Dr. Michael Osborne, Dr. Basil Rigas, Dr. John Sninsky, Dr. Mark Sobel, Dr. Steven Sommer, and Dr. Thierry Soussi). Please see letters of collaboration in the overview section of this program project grant.

**F. TIMETABLE****Task 1. Developing a multiplex polymerase chain reaction/ligase detection reaction (PCR/LDR) system for detection of point mutations in tumors.**

- a. Designing and synthesizing PCR and LDR primers for codons V157, R175, R248, R273, and R282. Testing these primers using PCR/LDR to identify cancer causing mutations in the p53 tumor suppressor gene in a variety of known cell lines and touch prep DNA samples. Months 1-18.
- b. Developing a multiplex PCR/LDR assay to simultaneously detect possible mutations in any one of 24 to 40 codons in the p53 gene in frozen and fixed primary breast tumors from the 100 to 200 breast cancer cases. Months 18-48.
- c. Designing and synthesizing PCR and LDR primers for detecting high risk HPV strains correlated with cervical carcinomas. Testing these primers using PCR/LDR to detect cancer causing HPV strains in clinical samples. Months 1-24.

**Task 2. Developing a multiplex ligase detection reaction/polymerase chain reaction (LDR/PCR) system for detection of gene amplifications and deletions in tumors.**

- a. Synthesizing ligase detection primers for the following genes: SOD, G6PD, p53, HER-2/neu, and int-2. Synthesizing "zip code primers". Testing LDR/PCR method with genomic DNA samples from normal, and trisomy 21 DNA's from human males and females. Months 1-18.
- b. Testing sensitivity of LDR/PCR on cell lines with known HER-2/neu and int-2 gene amplifications, and p53 deletions. Months 18-36.
- c. Using the LDR/PCR assay on frozen and fixed breast tumor material. Initial studies will assay 100 to 200 tumors to determine the feasibility of large scale studies correlating multiple genetic alterations with the clinical/biological behavior of breast cancers. Months 36-60.

**Task 3. Developing a polymerase chain reaction/restriction endonuclease/ligase detection reaction (PCR/RE/LDR) to detect and identify mutations at a sensitivity of 1 in  $10^6$  or  $10^7$  cells.**

- a. Improving (PCR/RE/LDR) using *TaqI* selection to detect a p53 codon 248 mutation at a sensitivity of 1 in  $10^6$  or  $10^7$  cells. Testing nucleotide analogues Q<sub>11</sub> and Q<sub>12</sub> for improved efficiency in converting to a *TaqI* site. Testing proofreading polymerases for potentially higher sensitivity in detection of cancer mutations. Months 1-24.
- b. Extending (PCR/RE/LDR) technology to include conversion of breast cancer p53 "hot spots" codons V157, R175, R273, and R282 into *TaqI* sites. Months 18-48.
- c. Using PCR/RE/LDR to test for the presence of tumor cells in lymph nodes and bone marrow samples from patients with primary breast tumors containing mutations in the 5 hot spots (as identified in Task 1b). In this initial study, the presence of micrometastases, will be correlated with HER-2/neu and int-2 gene amplification in the primary tumor (as identified in Task 2c). Months 36-60.
- d. Optimizing PCR/RE/LDR for converting DNA sequence to other restriction sites such as *Tth* 111 (Y163), *AlwNI* (L194 and R280), *BstXI* (M237, H179), *MwoI* (C242), and *BsII* (R249). Testing additional convertides available from project 3. In collaboration with Project 1, testing these primers for detecting mutations in lung and colon biopsies. Months 36-60.
- e. If preliminary results are successful, using PCR/RE/LDR on blood samples for detecting early relapse. Months 36-60.

**G. HUMAN SUBJECTS / VERTEBRATE ANIMALS:** Not applicable

**H. CONSULTANTS/COLLABORATORS:** Letters and Biographical Sketches for collaborators are attached in the overview section of this program project grant.

**I. CONSORTIUM/CONTRACTUAL ARRANGEMENTS:** None

**J. LITERATURE CITED.**

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## **Project 3.**

### **Design and Synthesis of Nucleotide Analogues**

**Project Leader: Donald Bergstrom  
Purdue University**

**Project Co-Leader: Robert P. Hammer  
Louisiana State University**



DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. DO NOT EXCEED THE SPACE PROVIDED.

A high sensitivity mutation detection system must be able to detect changes in any gene sequence. To be able to accomplish this, wild type DNA sequence corresponding to a mutation needs to be converted to a restriction enzyme site so repeated PCR amplification followed by digestion with the restriction enzyme removes the normal sequence while selectively amplifying the mutant sequence. This amplification, known as PCR/RE/LDR aims to detect one cancer mutation in  $10^6$  normal cells.

The goal of this project is to design and synthesize nucleotide analogues which facilitate sequence conversion. "Convertides" are nucleoside analogues which pair to one or more of the natural bases in an initial primer hybridization. More importantly, convertides also function as a degenerate template allowing for insertion of a different base during subsequent rounds of polymerase amplification. There are twelve possible nucleotide conversions which should be achieved.

To accomplish our goal we will work towards the following specific aims: (i) The synthesis of deoxyribonucleoside analogues to be used as convertides. Eight deoxyribonucleoside analogues, Q<sub>2</sub>, Q<sub>5</sub>, Q<sub>6</sub>, and Q<sub>9</sub>-Q<sub>13</sub>, have been previously described. We have already designed nine additional modified deoxyribonucleosides, Q<sub>1</sub>, Q<sub>3</sub>, Q<sub>4</sub>, Q<sub>7</sub>, Q<sub>8</sub>, and Q<sub>14</sub>-Q<sub>17</sub>. All 17 deoxyribonucleosides analogues will be synthesized in our laboratories. (ii) Preparation of dimethoxytrityl (DMT)-protected derivatives of all the convertides for incorporation into oligonucleotides. In the middle of an oligonucleotide, DMT-convertide phosphoramidites will be used. At the 3' position this will be accomplished by attaching the 3'-hydroxyl of the protected convertide to a long chain alkyl amine-CPG support. (iii) Testing of convertides for use in the mutation detection techniques. Starting with Q<sub>2</sub>, Core B will test convertide oligonucleotides as a means of increasing the specificity of mutation detection and as universal bases for polymorphic sites. (iv) Synthesizing and incorporating 5-propynyluridine into DNA or PNA "zip codes". In addressable arrays, this will be tested for optimizing the T<sub>m</sub> of the zip codes/complementary zip code duplexes (Project 5 and Core B).

PERSONNEL ENGAGED ON PROJECT, INCLUDING CONSULTANTS/COLLABORATORS. Use continuation pages as needed to provide the required information in the format shown below on *all* individuals participating in the project.

Name	BERGSTROM, Donald	Degree(s)	Ph.D.	Social Security #	REDACTED
Position Title	Professor	D.O.B.	REDACTED	Role on Project	Project Leader
Organization	Purdue Univ. School of Pharmacy & Pharm. Sciences			Department	Medicinal Chem.
Name	HAMMER, Robert	Degree(s)	Ph.D.	Social Security #	REDACTED
Position Title	Assistant Professor	D.O.B.	REDACTED	Role on Project	Project Co-leader
Organization	Louisiana State University			Department	Chemistry
Name	WANG, Guangyi	Degree(s)	Ph.D.	Social Security #	REDACTED
Position Title	Postdoctoral Fellow	D.O.B.	REDACTED	Role on Project	
Organization	Purdue University			Department	Medicinal Chem.
Name	ZHANG, Peiming	Degree(s)	Ph.D.	Social Security #	REDACTED
Position Title	Postdoctoral Research Assistant	D.O.B.	REDACTED	Role on Project	
Organization	Purdue University			Department	Medicinal Chem.
Name	COTHERN, Melissa	Degree(s)	B.S.	Social Security #	REDACTED
Position Title	Graduate Student	D.O.B.	REDACTED	Role on Project	Synthetic Chem.
Organization	Louisiana State University			Department	Chemistry
Name		Degree(s)		Social Security #	
Position Title		D.O.B.		Role on Project	
Organization				Department	
Name		Degree(s)		Social Security #	
Position Title		D.O.B.		Role on Project	
Organization				Department	

DD

Principal Investigator/Program Director (Last, first, middle): F. BARANY, Ph.D.

## DETAILED BUDGET FOR INITIAL BUDGET PERIOD

FROM

THROUGH

## DIRECT COSTS ONLY

94/12/01

95/11/30

PERSONNEL (Applicant Organization Only)					DOLLAR AMOUNT REQUESTED (omit cents)		
NAME	ROLE ON PROJECT	TYPE APPT. (months)	% EFFORT ON PROJ.	INST. BASE SALARY	SALARY REQUESTED	FRINGE BENEFITS	TOTALS
Donald E. Bergstrom	Principal Investigator	12	10				
Guangyi Wang	Post-Doc Fellow	12	100				
Peiming Zhang	Research Scientist	12	50				
<b>PROJECT 3</b>							
<b>SUBTOTALS</b>					\$37,107	\$7,198	\$44,305
<b>CONSULTANT COSTS</b>							\$0
<b>EQUIPMENT (Itemize)</b>							\$0
<b>SUPPLIES (Itemize by category)</b>							
Chemicals & Solvents \$3,000							
DNA Synthesis Reag \$4,000							
Chromatog. & Electroph. \$3,000							
Miscellaneous supplies \$3,000							\$13,000
<b>TRAVEL</b>							
One trip per year for P.I. to present results \$1,200							\$1,200
<b>PATIENT CARE COSTS</b>							
INPATIENT							\$0
OUTPATIENT							\$0
<b>ALTERATIONS AND RENOVATIONS (Itemize by category)</b>							\$0
<b>OTHER EXPENSES (Itemize by category)</b>							
See Following Page \$7,500							\$7,500
<b>SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD</b>							\$66,005
<b>CONSORTIUM/CONTRACTUAL COSTS</b>							
<b>DIRECT COSTS</b>					<b>TOTAL</b>		\$33,663
INDIRECT COSTS 51%							
<b>TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD</b>							\$99,668

**BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD  
DIRECT COSTS ONLY**

PROJECT 3

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED			
			2nd	3rd	4th	5th
<b>PERSONNEL:</b>						
<i>Salary &amp; fringe benefits</i>						
<i>Applicant organization only</i>		\$44,305	\$46,077	\$47,920	\$49,837	\$51,830
<b>CONSULTANT COSTS</b>		\$0	\$0	\$0	\$0	\$0
<b>EQUIPMENT</b>		\$0	\$2,000	\$2,000	\$2,000	\$2,000
<b>SUPPLIES</b>		\$13,000	\$13,520	\$14,061	\$14,623	\$15,208
<b>TRAVEL</b>		\$1,200	\$1,248	\$1,298	\$1,350	\$1,404
<b>PATIENT CARE COSTS</b>	<b>INPATIENT</b>	\$0	\$0	\$0	\$0	\$0
	<b>OUTPATIENT</b>	\$0	\$0	\$0	\$0	\$0
<b>ALTERATIONS AND RENOVATIONS</b>		\$0	\$0	\$0	\$0	\$0
<b>OTHER EXPENSES</b>		\$7,500	\$7,800	\$8,112	\$8,436	\$8,773
<b>SUBTOTAL DIRECT COSTS</b>		\$66,005	\$70,645	\$73,391	\$76,246	\$79,215
<b>CONSORTIUM/ CONTRACTUAL COSTS</b>		\$33,663	\$35,010	\$36,410	\$37,866	\$39,381
<b>TOTAL DIRECT COSTS</b>		\$99,668	\$105,655	\$109,801	\$114,112	\$118,596
<b>TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD</b>						<b>\$547,832</b>

(Item 8a)-&gt;

**JUSTIFICATION** (Use continuation pages if necessary):

**From Budget for Initial Period:** Describe the specific functions of the personnel, collaborators, and consultants and identify individuals with appointments that are less than full time for a specific period of the year, including VA appointments.

**For All Years:** Explain and justify purchase of major equipment, unusual supplies requests, patient care costs, alterations and renovations, tuition remission, and donor/volunteer costs.

**From Budget for Entire Period:** Identify with an asterisk (\*) on this page and justify any significant increase or decrease in any category over the initial budget period. Describe any change in effort of personnel.

**For Competing Continuation Applications:** Justify any significant increases or decreases in any category over the current level of support.

**INITIAL BUDGET PERIOD:**

**Personnel:** Dr. Bergstrom will coordinate and oversee the entire project, and direct the research on modified nucleoside synthesis at Purdue University. Dr. Wang, a postdoctoral research associate, will work on developing synthetic routes to new nucleoside analogs. Dr. Zhang, whose support is provided by the Walther Cancer Institute, Indianapolis, Indiana, will synthesize nucleoside analogs already under development at Purdue University. Both Dr. Wang and Dr. Zhang will synthesize nucleoside phosphoramidites, nucleoside modified controlled pore glass, and oligonucleotides containing the modified nucleosides. Dr. Wang will carry out biophysical (T<sub>m</sub> studies) measurements on oligonucleotides containing modified nucleosides. Institutional base salary represents a calculated average salary for the project period which may

transcend multiple Purdue fiscal years with raise factors included. Faculty and other salaries are calculated with a 4% fiscal year increase per university guidelines.

*Supplies and Other Expenses:* HPLC Service Contract - \$2,000; Reprint charges - \$500; NMR spectra - \$3,000; Mass spectra - \$600; Telephone - \$400; Quanta & SYBYL maintenance - \$1,000. Supplies are calculated with a 6% inflationary factor. All categories are calculated with a 5% fiscal year increase. All synthetic intermediates will be characterized by  $^1\text{H}$  and  $^{13}\text{C}$  NMR. Phosphorous-containing compounds will be characterized also by  $^{31}\text{P}$  NMR. Estimated cost for obtaining spectra is \$20 per nuclei. Estimated cost of NMR spectra is \$3,000 per year. The estimated cost for DNA synthesis reagents is based on the projected synthesis of 100 oligonucleotides 20-24 bases in length. The cost of a service contract for a Beckman HPLC system (\$2,000) is included in the budget, since purification of oligonucleotides will make extensive use of this instrument. The maintenance contract for the Quanta and SYBYL molecular modeling software is included, because we use these programs for modeling modified bases in DNA duplexes.

*Travel:* Funds are requested for travel to attend one domestic meeting per year.

*Consortium/Contractual Costs:* This represents the Purdue University Negotiated Indirect Cost rate of 51% of modified direct costs (total direct costs - major equipment costs).

*Budget for Years 2-5:* The effort and personnel devoted to the project will remain constant throughout the five years of the grant. All budget categories are calculated with a 4% fiscal year increase.

DD

Principal Investigator/Program Director (Last, first, middle): **F. BARANY, Ph.D.**  
**DETAILED BUDGET FOR INITIAL BUDGET PERIOD**  
**DIRECT COSTS ONLY**

FROM 94/12/01 THROUGH 95/11/30

PERSONNEL (Applicant Organization Only)		TYPE APPT. (months)	% EFFORT ON PROJ.	INST. BASE SALARY	DOLLAR AMOUNT REQUESTED (omit cents)		
NAME	ROLE ON PROJECT				SALARY REQUESTED	FRINGE BENEFITS	TOTALS
Robert P. Hammer (AY)	Principal Investigator	9	10				
Robert P. Hammer (ss)	Co-Investigator	3	66.7				
To be appointed	Post-Doc Associate	12	100				
<b>PROJECT 3</b>							
SUBTOTALS					\$34,000	\$6,800	\$40,800
CONSULTANT COSTS							\$0
EQUIPMENT (Itemize)							
Rotary Evaporator		\$3,065					
Cold Trap for Centrivap		\$1,600					\$4,665
SUPPLIES (Itemize by category)							
HPLC columns/solvents		\$3,000					
DNA synthesis chemicals		\$5,000					
Chemicals and other consumables		\$6,000					\$14,000
TRAVEL							
One trip per year for P.I. to present results				\$1,200			\$1,200
PATIENT CARE COSTS		INPATIENT					\$0
		OUTPATIENT					\$0
ALTERATIONS AND RENOVATIONS (Itemize by category)							\$0
OTHER EXPENSES (Itemize by category)							
See Following Page		\$4,500					\$4,500
<b>SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD</b>							<b>\$65,165</b>
CONSORTIUM/CONTRACTUAL COSTS							
DIRECT COSTS					TOTAL		\$27,225
INDIRECT COSTS		45%					
<b>TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD</b>							<b>\$92,390</b>

**BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD  
DIRECT COSTS ONLY**

**PROJECT 3**

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED			
			2nd	3rd	4th	5th
<b>PERSONNEL:</b>						
<i>Salary &amp; fringe benefits</i>						
<i>Applicant organization only</i>		\$40,800	\$42,432	\$44,129	\$45,894	\$47,730
<b>CONSULTANT COSTS</b>		\$0	\$0	\$0	\$0	\$0
<b>EQUIPMENT</b>		\$4,665	\$2,000	\$2,000	\$2,000	\$2,000
<b>SUPPLIES</b>		\$14,000	\$14,560	\$15,142	\$15,748	\$16,378
<b>TRAVEL</b>		\$1,200	\$1,248	\$1,298	\$1,350	\$1,404
<b>PATIENT CARE COSTS</b>	<b>INPATIENT</b>	\$0	\$0	\$0	\$0	\$0
	<b>OUTPATIENT</b>	\$0	\$0	\$0	\$0	\$0
<b>ALTERATIONS AND RENOVATIONS</b>		\$0	\$0	\$0	\$0	\$0
<b>OTHER EXPENSES</b>		\$4,500	\$4,680	\$4,867	\$5,062	\$5,264
<b>SUBTOTAL DIRECT COSTS</b>		\$65,165	\$64,920	\$67,436	\$70,054	\$72,776
<b>CONSORTIUM/ CONTRACTUAL COSTS</b>		\$27,225	\$28,314	\$29,447	\$30,625	\$31,850
<b>TOTAL DIRECT COSTS</b>		\$92,390	\$93,234	\$96,883	\$100,679	\$104,626
<b>TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD</b>						<b>\$487,812</b>

(Item 8a)->

**JUSTIFICATION (Use continuation pages if necessary):**

**From Budget for Initial Period:** Describe the specific functions of the personnel, collaborators, and consultants and identify individuals with appointments that are less than full time for a specific period of the year, including VA appointments.

**For All Years:** Explain and justify purchase of major equipment, unusual supplies requests, patient care costs, alterations and renovations, tuition remission, and donor/volunteer costs.

**From Budget for Entire Period:** Identify with an asterisk (\*) on this page and justify any significant increase or decrease in any category over the initial budget period. Describe any change in effort of personnel.

**For Competing Continuation Applications:** Justify any significant increases or decreases in any category over the current level of support.

**INITIAL BUDGET PERIOD:**

**Personnel:** Robert P. Hammer will act as Co-leader of the project and direct nucleotide analogue synthesis at Louisiana State University. Melissa Cothorn (not listed above) is a graduate assistant who just joined the Co-leader's laboratory. She has received a prestigious Board of Regents' Fellowship and will fully supported for the next 3 years and she will be working full time on this project. The postdoctoral associate will have experience in the synthesis of mononucleosides and oligonucleotides. The Co-leader, graduate assistant, and the postdoctoral associate (100%) will be performing the organic synthesis of mononucleoside analogs, incorporation of these analogs into oligonucleotides and, when appropriate, biophysical measurements on oligonucleotides containing modified nucleosides.

*Equipment:* Dr. Hammer has been at Louisiana State University since August 1992 and received ample funds and departmental support to equip his laboratory. The demands of the proposed work will, however, put pressure on the current capabilities. The requested rotary evaporator (\$3,065) will be used exclusively for the proposed work, and the cold trap (\$1,600) will be attached to the currently existing Labconco Centrivap concentrator, which will make it more efficient for concentration of oligonucleotide samples.

*Supplies:* Preparation of nucleoside phosphoramidites requires the use of relatively expensive intermediates. As rapid progress in preparation of nucleoside analogs and their respective oligonucleotides is essential to the overall goal of LDR application to cancer detection, advanced intermediates will be purchased whenever possible. The estimated cost of \$6,000 covers nucleoside and sugar starting reagents, solvents, and glassware as well as other consumables. DNA synthesis reagents are purchased at contract rates. The estimate of \$5,000 covers costs of natural phosphoramidites, CPG supports, solvents, and reagents for the Pharmacia Gene Assembler in our laboratory. Oligonucleotides will be purified by anion-exchange and/or reversed-phase HPLC; \$3,000 covers the costs of solvents, minor HPLC repairs, and new columns (~1 year lifetime with vigorous use).

*Travel:* One trip to a National meeting for Dr. Hammer or one of his associates to present results.

*Other Expenses:* Communication with Project Team will be essential for rapid progress. Thus, the \$1,000 budgeted reflects the cost of phone and facsimile communication that will be used in keeping other Team members up to date on our most recent results. Characterization of the organic intermediates requires the use of Departmental facilities and outside elemental analysis (\$3,000). Copying and page charges for publications resulting from this work are estimated at \$500.

*Consortium/Contractual Costs:* This reflects the LSU Negotiated Indirect Cost rate of 45% of modified direct costs (total direct costs – major equipment costs).

#### **SUBSEQUENT YEARS' BUDGETS (2-5)**

An additional \$2,000 per year is requested for equipment. Personpower on the project will remain the same throughout the project. Increases in budget for salary, supplies, etc. reflects a 4% inflationary adjustment.

**BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD  
DIRECT COSTS ONLY**

PROJECT 3

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED			
			2nd	3rd	4th	5th
<b>PERSONNEL:</b>						
<i>Salary &amp; fringe benefits</i>						
<i>Applicant organization only</i>		\$85,105	\$88,509	\$92,049	\$95,731	\$99,560
<b>CONSULTANT COSTS</b>		\$0	\$0	\$0	\$0	\$0
<b>EQUIPMENT</b>		\$4,665	\$4,000	\$4,000	\$4,000	\$4,000
<b>SUPPLIES</b>		\$27,000	\$28,080	\$29,203	\$30,371	\$31,586
<b>TRAVEL</b>		\$2,400	\$2,496	\$2,596	\$2,700	\$2,808
<b>PATIENT CARE COSTS</b>	<b>INPATIENT</b>	\$0	\$0	\$0	\$0	\$0
	<b>OUTPATIENT</b>	\$0	\$0	\$0	\$0	\$0
<b>ALTERATIONS AND RENOVATIONS</b>		\$0	\$0	\$0	\$0	\$0
<b>OTHER EXPENSES</b>		\$12,000	\$12,480	\$12,979	\$13,498	\$14,038
<b>SUBTOTAL DIRECT COSTS</b>		\$131,170	\$135,565	\$140,827	\$146,300	\$151,992
<b>CONSORTIUM/ CONTRACTUAL COSTS</b>		\$60,888	\$63,324	\$65,857	\$68,491	\$71,231
<b>TOTAL DIRECT COSTS</b>		\$192,058	\$198,889	\$206,684	\$214,791	\$223,223
<b>TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD</b>						<b>\$1,035,645</b>

(Item 8a)-&gt;

**JUSTIFICATION (Use continuation pages if necessary):**

**From Budget for Initial Period:** Describe the specific functions of the personnel, collaborators, and consultants and identify individuals with appointments that are less than full time for a specific period of the year, including VA appointments.

**For All Years:** Explain and justify purchase of major equipment, unusual supplies requests, patient care costs, alterations and renovations, tuition remission, and donor/volunteer costs.

**From Budget for Entire Period:** Identify with an asterisk (\*) on this page and justify any significant increase or decrease in any category over the initial budget period. Describe any change in effort of personnel.

**For Competing Continuation Applications:** Justify any significant increases or decreases in any category over the current level of support.

**Total 5yr budgets for Project 3**



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**RESOURCES AND ENVIRONMENT**

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FACILITIES: Mark the facilities to be used at each performance site listed in Item 9, Face Page, and briefly indicate their capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Use "Other" to describe the facilities at any other performance sites listed in Item 9 on the Face Page and at sites for field studies. Use continuation pages if necessary. Include an explanation of any consortium/contractual arrangements with other organizations.

- ☒ **Laboratory:** New laboratory facilities (May 1993) totaling 2500 ft<sup>2</sup> with bench space and 10 hoods for synthetic chemistry are located in the Hansen Life Sciences Research Building at Purdue University.
- ☐ **Clinical:**
- ☐ **Animal:**
- ☒ **Computer:** Computer facilities are available within the Department for data analysis, searching of nucleotide sequences, and molecular modeling. The AIDS Center Computational Chemistry Facility includes an Evans and Sutherland PS390, a VAX, a Silicon Graphics 4D/120, a Silicon Graphics 4D/20, and an IRIS 3030 workstation. Both SYBYL and Polygen software are used for modeling oligonucleotide structures.
- ☒ **Office:** The P.I.'s office (150 ft<sup>2</sup>) with two desks and a conference table is located in the center of the laboratory.
- ☐ **Other ( )::**

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MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

See list on following page.

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ADDITIONAL INFORMATION: Provide any other information describing the environment for the project. Identify support services such as consultant, secretarial, machine shop, and electronics shop, and the extent to which they will be available to the project.

Within the same buliding are located service laboratories for DNA synthesis and sequencing, protein synthesis and sequencing, a hybridoma and antibody production facility, and the analytical cytology laboratory.

## Major Equipment:

### A. Located in the laboratory space of the Principal Investigator

1. pH Meter, Radiometer PHM 63
2. Fraction collectors, Gilson FC-80M, FC-100, and FC-203
3. Microanalytical balance, Cahn 21
4. Semimicro balances (2), Mettler H33AR and Sartorius A200S
5. Electronic balance, Mettler ME 360
6. DNA synthesizer, Milligen/Bioscience 8700
7. Inert atmosphere system, Vacuum Atmospheres
8. Recording integrator, Shimadzu C-R3A.
9. Melting point apparatus, Buchi 510
10. Rotary evaporators (4), Buchi, Yamato
11. Freeze dryer, Virtis
12. UV monitors, Gilson 260 and 111B
13. High vacuum system with two-stage oil diffusion pump, Edwards
14. FT-IR, Nicolet Magna-IR 550 (CsI)
15. Gradient HPLC Systems (2), Beckman
  - Autosampler model, 507
  - Diode array detector model, 168
  - Variable wavelength UV-Vis detector
  - IBM Model 70-386 microcomputer
  - System Gold software
16. Liposome preparation apparatus, Lipoprep™
17. Ion analyzer, Orion 920 (with fluoride ion electrode)
18. Evaporator centrifuge, Savant Speedvac
19. UV-VIS spectrophotometer, Shimadzu 260, with electronic temperature controlled cell
20. Constant temperature baths, Lauda RM6 (2)
21. Centrifuge, high speed, Centra 4B
22. Microcomputers, Macintosh 512, SE, and IICx linked to a Laserwriter via Appletalk
23. Electrophoresis apparatus with power supply
24. Transilluminator System, Fotodyne FCR-10
25. Photochemical Apparatus, Southern New England

### B. Departmental Instrumentation

All major modern instrumentation and support facilities are available within the Department. These include mass spectrometers (Kratos MS-50 and MS-25), NMR spectrometers (Varian VXR-500 and Bruker AXR-300), FTIR spectrometers, fluorescence spectrometers, centrifuge, and scintillation counters.

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**RESOURCES AND ENVIRONMENT**

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FACILITIES: Mark the facilities to be used at each performance site listed in Item 9, Face Page, and briefly indicate their capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Use "Other" to describe the facilities at any other performance sites listed in Item 9 on the Face Page and at sites for field studies. Use continuation pages if necessary. Include an explanation of any consortium/contractual arrangements with other organizations.

☒ **Laboratory:**

Dr. Hammer has two 700 sq. ft. laboratories (639 and 710 Choppin Hall) each equipped with 3 fume hoods. The laboratories have been set-up for doing organic synthesis and includes a double-manifold (vacuum, inert gas) for performing air and moisture sensitive reactions, flash chromatography equipment for purifying organic intermediates, and stills for purification and drying of solvents immediately before use. Small equipment includes: 2 Büchi model RE-121 rotary evaporators, 1 Büchi model B-171 Vacobox PTFE pump for use with up to two rotary evaporators, 2 high vacuum pumps, 2 analytical balances, 1 top loading balance, and 1 Labconco centrifugal heated evaporator for concentration of oligonucleotide samples (requires a dedicated vacuum pump). Major equipment items in the co-PI's laboratories are a Waters Model 600E HPLC system (0-20 mL/min) complete with a model 717 Autosampler, model 486 variable wavelength (190-700 nm) detector, column heater and column switching valve; and a Pharmacia Gene Assembler Plus oligonucleotide synthesizer with on-line detection of trityl cations for immediate synthesis evaluation and coupling yield determination.

☐ **Clinical:**☐ **Animal:**☒ **Computer:**

The office has an Apple Macintosh IIci (5 MB RAM, 240 MB hard drive) on the University Ethernet system; the laboratory has a Apple Macintosh Classic computer connected to the departmental Appetalk network. Departmental Computing and Molecular Modeling facilities (MCAF) will be updated in Spring 1994 with latest work stations and computers. It currently has a cluster of 5 microVAX 3000 series computers and several high resolution graphics terminal including an Evans and Sutherland ESV50, which has several molecular modeling and mechanics/dynamics packages including SYBYL and Macromodel.

☒ **Office:**

The PI also has a 140 sq. ft. office (742 Choppin Hall) with a Apple Macintosh IIci (5 MB RAM, 240 MB hard drive).

☐ **Other ( ):**

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MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

All equipment needed for this work is located in Choppin Hall. NMR's: For walk-up use, a Bruker 250 MHz equipped with  $^1\text{H}/^{13}\text{C}/^{31}\text{P}$  probe and a Bruker 200 MHz for  $^1\text{H}/^{13}\text{C}$ ; Two high-field instruments for more demanding experiments, Bruker 400 MHz and 500 MHz machines located in College NMR Facility. IR: Perkin Elmer model 1760X FTIR. UV/VIS/NIR: Aviv model 14DS equipped with a 5 cell thermoelectrically cooled sample holder with software for automated data collection and data handling/presentation. Circular Dichroism Spectroscopy: Aviv CD spectrometer with thermoelectrically cooled sample holder and computer interface for data acquisition and handling. Mass Spectrometry: Finnigan TSQ70 GC/MS with electron impact, chemical ionization and fast atom bombardment sources; Bio-Ion  $^{252}\text{Cf}$  plasma desorption mass spectrometer for low resolution time-of-flight measurements up to 15,000 amu. X-ray Facility: Two Enraf-Nonius CAD4 diffractometers, one with copper radiation and the other with molybdenum radiation.

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ADDITIONAL INFORMATION: Provide any other information describing the environment for the project. Identify support services such as consultant, secretarial, machine shop, and electronics shop, and the extent to which they will be available to the project.

The department has machine, glass blowing, and electrical shops with expert technicians for in house design and repair of equipment. Secretarial assistance is available from the main office and student workers (fellowship or financial aid recipients). Drafting and graphics services are also provided at minimal cost.

## A. SPECIFIC AIMS

The syntheses of nucleoside analogs, designed to function as base transposing agents, herein called *convertides* (Q<sub>1</sub>-Q<sub>17</sub>; see section D, Scheme 3 for structures and potential hydrogen bonding schemes), are proposed. "Convertides" are nucleoside analogues which pair to one or more of the natural bases in an initial primer hybridization ("read step"). More importantly, convertides also function as a degenerate template allowing for insertion of a different base during subsequent rounds of polymerase amplification ("write step"). For performing the 12 possible base conversions, 8 known deoxyribonucleoside analogs (Q<sub>2</sub>, Q<sub>5</sub>, Q<sub>6</sub>, Q<sub>9</sub>-Q<sub>13</sub>) and 9 newly-designed, modified deoxyribonucleosides (Q<sub>1</sub>, Q<sub>3</sub>, Q<sub>4</sub>, Q<sub>7</sub>, Q<sub>8</sub>, Q<sub>14</sub>-Q<sub>17</sub>) will be utilized. Dimethoxytrityl(DMT)-protected derivatives of all the convertides will be prepared and incorporated into oligonucleotides (sequences specified by Core 2 participants) either at the 3'-end by attachment of the free 3'-hydroxyl of the protected convertide to a long chain alkyl amine-CPG support or alternatively in the middle of a chain by use of DMT-convertide phosphoramidites.

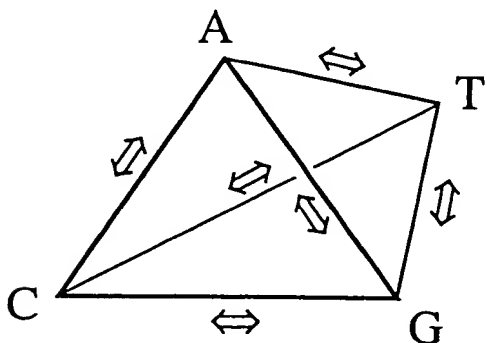
In concert with Project 4, convertides (initially Q<sub>2</sub>), will be incorporated near the discriminating base of an LDR primer as a means to enhance the specificity of cancer mutation detection. Also, application of these nucleotide analogs as universal base-pairs to help avoid problems in DNA detection caused by polymorphism surrounding the discriminating nucleotides (Projects 1, 2 and 4) will be explored.

## B. BACKGROUND AND SIGNIFICANCE

In order to most efficiently bring about the base conversions proposed by Dr. Francis Barany in Project 2, nucleoside analogs are required which are capable of forming DNA polymerase-recognizable base pairs to more than one natural base. These analogs, when positioned at the 3'-end of a primer are designed to allow hybridization and DNA polymerase-mediated extension against one specific base (defined as the "read" step) but then to function as a template for a different base in a subsequent round of replication ("write"). Since the final result is the conversion of one base in a sequence to another, the analogs have been defined as *Convertides*. A major goal of this project is to design and synthesize new, as well as existing, nucleoside analogs to function as selective convertides. Because there are 12 different base conversions (Scheme 1), ideally 12 separate nucleoside analogs (convertides) should be developed, each specific for one conversion. Each convertide presents a unique design problem.

### Scheme 1

There are twelve different base conversions each of which could conceptionally be mediated by a unique convertide.



A ⇒ C	C ⇒ A
A ⇒ G	G ⇒ A
A ⇒ T	T ⇒ A
C ⇒ G	G ⇒ C
C ⇒ T	T ⇒ C
G ⇒ T	T ⇒ G

A short explanation of the relationship between convertide mediated base conversions and site-directed mutagenesis may help to explain the rationale behind the design of these analogs. In site-directed mutagenesis, a primer is constructed in which one or more bases is replaced by a mismatch to the target sequence. This mismatch is chosen to correspond to the complement of the base that one wishes to insert.

Typically the mismatch is located at some position in the middle of the sequence. Following extension of the primer, a second round of replication yields a sequence in which the desired base is inserted in place of the original base. This technique is usually successful because the mismatch is upstream from the 3'-end of the primer and does not interfere with DNA polymerase binding and chain extension. Convertide-mediated base conversion works differently. The convertide is placed at the 3'-end of the primer and extended with a thermally stable polymerase (e.g. *Taq* polymerase), which typically have low tolerance for base mismatches. Convertides are designed to pair with two or more bases with similar affinity and to as closely as possible occupy space in a duplex like the natural base-pairs. During the PCR replication of the first cycle the 3'-convertide is required to read one base and then preferentially insert a different nucleoside during the second cycle. This is illustrated for the G≡C to A=T conversion shown in Scheme 2.

Recent literature on unnatural and "universal" nucleoside analogs provides a starting point for convertide design. There have been numerous reports of nucleoside analogs designed to pair with more than one of the four primary DNA bases [1-8]. The most extensively studied example is 2'-deoxyinosine which has been used as a putative "universal" nucleoside in oligonucleotide probes and primers since its introduction in 1985 [9]. Deoxyinosine (denoted as **Q<sub>11</sub>** in this proposal) is believed to function as a "universal nucleoside" because it both contains the necessary polarized planar aromatic  $\pi$ -system for base stacking interactions and a hydrogen bond acceptor site (O<sup>6</sup>) adjacent to an acidic hydrogen (H-N<sup>1</sup>) for hydrogen bonding to each of the four deoxyribonucleosides dA, dC, dG, and T. Structural studies on deoxyinosine modified oligonucleotides show that dI can base-pair to dC [10], dA [11], T [12,13] and dG[14]. However, deoxyinosine prefers to base pair to dC. The dI-dC base pair is 1 to 3 kcal/mol more stable than any of the other base pairs containing dI [15,16]. The next most stable is dI-dA, while dI-dG and dI-dT are significantly less stable. Consequently, dI is a good candidate for a convertide because it preferentially should write a single nucleoside, dC, but may read others, such as dA. Of course, predictions such as this based on  $T_m$  data are of limited value since the most important factor, recognition by the DNA polymerase may not be directly related to binding affinity. The dI-dA base pair may be relatively stable because one of the bases (dA) assumes a syn conformation and hydrogen bonds through N-7.

### C. PRELIMINARY STUDIES

**Nucleoside Design** – This project represents an integrated effort of two separate research programs. Historically, the concept of convertides and the design of at least half of the new nucleoside analogs described in this proposal resulted from the combined effort of the program project principal investigator, Dr. Francis Barany, and Dr. Robert Hammer, co-principal investigator on this project. Dr. Bergstrom was added to the project at a later stage because of his laboratory's involvement and experience in the design and synthesis of universal nucleoside analogs. The current proposal represents an integration of the ideas and concepts of convertides as originated by Barany and Hammer, and the concept of five-membered ring heterocycles as universal bases under development in the Bergstrom laboratory. The application of two separate research groups to this problem is justified because of the scope of the synthetic problem. Unlike most conventional nucleoside analog projects, which involve the synthesis of a family of modified nucleosides of similar structures (for example, in establishing structure-activity profiles for antiviral nucleoside analogs), the technology described in the overall program project would benefit significantly from the availability of twelve different nucleoside analogs, each specific for the metamorphoses shown in Scheme 1. Many different heterocyclic systems, including both N- and C-nucleosides, as well as an exceptionally broad range of synthetic methodologies and strategies are represented in this proposal. A cooperative and synergistic effort by two strong synthetic research groups significantly increases the potential for success. The principal preliminary accomplishment of the Hammer and Bergstrom research groups has been in the area of molecule design, which will become apparent in section D. However, both synthetic and biochemical studies, which have direct bearing on this proposal, have been accomplished.

The proposed 2'-deoxynucleoside analogs have the following properties: (1) The natural base is replaced with a heteroaromatic base which can participate in favorable " $\pi$ -stacking" interactions; (2) The replacement base has substituents which can form Watson-Crick-like hydrogen bonds in several different

ways; and (3) At least one of the potential base-pairs formed with complementary purines or pyrimidines conforms to the steric and geometric parameters for a normal B-type double helix. The compounds proposed for this study are listed in Table 1. Structures for these compounds and predictions of the role of each analog in base-pairing to the natural bases is illustrated in Scheme 3 and in Table 4. An important criteria for the modified nucleosides designed for this study was that each be capable of assuming either multiple tautomeric forms or exist in multiple conformations in order to present more than one hydrogen bonding pattern.

Table 1

## Q Nomenclature

Q1	1-(2'-deoxy-β-D-ribofuranosyl)imidazole-4-carboxamide
Q2	1-(2'-deoxy-β-D-ribofuranosyl)-3-nitropyrrole
Q3	5-(2'-deoxy-β-D-ribofuranosyl)-2-pyrimidinone
Q4	5-(2'-deoxy-β-D-ribofuranosyl)-2-thiopyrimidine
Q5	3-(2'-deoxy-β-D-ribofuranosyl)-2,7-dioxypyrido[2,3- <i>d</i> ]pyrimidine
Q6	5-amino-1-(2'-deoxy-β-D-ribofuranosyl)imidazole-4-carboxamide
Q7	5-amino-3-(2'-deoxy-β-D-ribofuranosyl)-2,7-dioxypyrimido[4,5- <i>d</i> ]pyrimidine
Q8	3-(2'-deoxy-β-D-ribofuranosyl)-2,7-dioxo-5-thiopyrimido[4,5- <i>d</i> ]pyrimidine
Q9	1-(2'-deoxy-β-D-ribofuranosyl)-2-oxo-4-imidazoline-4-carboxamide
Q10	2'-deoxy-5-fluorouridine
Q11	2'-deoxyinosine
Q12	6-(2'-deoxy-β-D-ribofuranosyl)-6H,8H-3,4-dihydropyrimido[4,5- <i>c</i> ][1,2]oxazine-7-one
Q13	2-amino-7-(2'-deoxy-β-D-ribofuranosyl)-6-methoxyaminopurine
Q14	2-carbamoyl-4-(2'-deoxy-β-D-ribofuranosyl)imidazole
Q15	2-amino-1-(2'-deoxy-β-D-ribofuranosyl)imidazole-4-carboxamide
Q16	1-(2'-deoxy-β-D-ribofuranosyl)-2-methoxyaminoimidazole-4-carboxamide
Q17	4-amino-6-(2'-deoxy-β-D-ribofuranosyl)-2,7-dioxypyrido[2,3- <i>d</i> ]pyrimidine

Except for Q3 and Q4 (pyridones), Q10 (fluorouridine), Q11 (inosine) and Q13, in all of the proposed convertides, the natural base is replaced with either a 5-membered nitrogen heterocycle (pyrrole, Q2; imidazole, Q1, Q6, Q9, Q14-Q16) or a 6,6-fused heteroaromatic (pyridopyrimidine, Q5, Q17; pyrimido-oxazine, Q12; or pyrimidopyrimidines; Q7, Q8). In section D.1., a graphic description of potential base-pairing properties is provided for each of the convertides Q1 - Q17. One of these compounds, Q2 has been studied as potential universal nucleoside. A summary of the results of this study is presented below.

**Design of Q2 as a Universal Nucleoside** - For all of the convertides, except one (Q2), specificity is expected to be a result of hydrogen bonding pattern preferences. Q2 is a unique nucleoside analog which has the potential to function as a convertide despite its lack of a hydrogen donor site. Q2 was designed to maximize stacking interactions through attachment of a highly polar aprotic substituent to a highly polarizable heteroaromatic ring. 3-Nitropyrrole deoxyribonucleoside structurally and electronically resembles p-nitroaniline, derivatives of which are among the smallest known double-stranded DNA intercalators [17]. A charge distribution contour map of 3-nitropyrrole was generated in the software program SYBYL using the Del Re and Hückel methods to obtain partitioned σ- and π-charge contributions. The electronic distribution of 3-nitropyrrole was found to closely resemble the average charge distribution of A, C, G, and T. 3-Nitropyrrole deoxyribonucleoside (Q2) was subsequently synthesized and its structure confirmed by X-ray crystallography [18]. For incorporation into oligonucleotides, Q2 was transformed to a dimethoxytrityl-protected phosphoramidite, which was used with conventional protocols in an automated DNA synthesizer to construct modified oligonucleotides. Stepwise yields, evaluated by spectrophotometric monitoring of the dimethoxytrityl release at each cycle of synthesis, were no different from those obtained using phosphoramidites of conventional bases. Oligonucleotides designed to test the function of a potential universal nucleoside in both Sanger dideoxy-sequencing and PCR were synthesized.

**Sequencing and PCR Studies with Q<sub>2</sub>** – In collaboration with researchers at the University of Michigan, Q<sub>2</sub> containing oligonucleotides were tested first as primers for Sanger dideoxy sequencing using T7 DNA polymerase and then as primers for PCR amplification of specific sequences in a cDNA library. The results of these studies show that substantial numbers of nucleosides in a 17-mer primer can be replaced by 3-nitropyrrole 2'-deoxyribonucleoside (Q<sub>2</sub>) without loss of primer function. For example, in dideoxy sequencing, the primers 5'-CGT AAQ<sub>2</sub> CAQ<sub>2</sub> AAQ<sub>2</sub> ACQ<sub>2</sub> AT-3' and 5'-CGT AAT CAG Q<sub>2</sub>Q<sub>2</sub>Q<sub>2</sub> Q<sub>2</sub>Q<sub>2</sub>Q<sub>2</sub> AT-3' give perfectly readable sequencing ladders virtually identical to the control sequence 5'-CGT AAT CAG AAA ACA AT-3'. In contrast, control sequences, which on hybridization yield two or more natural base mismatches (for example C-T, and G-T in place of A-T) gave unreadable sequencing ladders. A more detailed description of these experiments and the results are outlined in a manuscript included in the appendix.

The Q<sub>2</sub> containing primers listed in Table 2 were used to amplify from total RNA using conventional PCR conditions. Total RNA was isolated from whole *Drosophila melanogaster* according to the method of Chirgwin et al. [19]. First strand cDNA was made from total RNA with AMV reverse transcriptase and oligoT at 42°C for 1 hour (100 µl, total reaction volume). The first strand synthesis was divided into four equal volumes and used for amplification with four different primers (Table 2). The primers included one with a perfect match (**primer 1**), one with Q<sub>2</sub> at four degenerate sites (**primer 2**), one with Q<sub>2</sub> at the third position in from the 3-end (**primer 3**) and, one with Q<sub>2</sub> at the 3'-end of the oligonucleotide (**primer 4**). PCR was done for 30 cycles (annealed at 48°C, extended at 72°C, and denatured at 95°C) using Taq polymerase in a total volume of 100 µl. The PCR products were analyzed by agarose gel electrophoresis using 10 µl of each reaction. The identity of the amplified sequence was verified by dideoxy sequence analysis and DNA blot hybridization using a radiolabeled probe internal to the PCR primers. All four primers led to successful amplification of the target sequence, although **primer 2** gave noticeably less amplification than the other sequences.

Table 2

Primer No.	Sequence					
1	5'-CGT	AAT	CAG	AAA	ACA	AT-3'
2	5'-CGT	AAQ <sub>2</sub>	CAQ <sub>2</sub>	AAQ <sub>2</sub>	ACQ <sub>2</sub>	AT-3'
3	5'-CGT	AAT	CAG	AAA	ACQ <sub>2</sub>	AT-3'
4	5'-CGT	AAT	CAG	AAA	ACA	AQ <sub>2</sub> -3'

**T<sub>m</sub> Measurements** – In order to determine if Q<sub>2</sub> was non-discriminatory in base-pairing to A, C, G, and T it was necessary to carry out DNA duplex melting studies. T<sub>m</sub> data for hybridization of the sequences 5'-C<sub>2</sub>T<sub>5</sub>X T<sub>5</sub>G<sub>2</sub>-3' and 5'-G<sub>2</sub>A<sub>5</sub>Y A<sub>5</sub>C<sub>2</sub>-3' was obtained for both Q<sub>2</sub> and 4-bromopyrazole deoxyribonucleoside (Table 3). An ideal universal nucleoside would show nearly equivalent T<sub>m</sub>'s with A, C, G, and T. Q<sub>2</sub> appears to be relatively non-discriminatory. T<sub>m</sub>'s extended over a narrow range (2.7°C) for oligonucleotides with Q<sub>2</sub> opposite A, C, G, and T. In contrast the T<sub>m</sub>'s for the duplexes in which various combinations of the normal bases were placed opposite one another varied over a much wider range. For example, as shown in the table, cytosine containing base pairs showed T<sub>m</sub>'s extending over a 15 °C range. Preliminary data on deoxyinosine containing oligonucleotides indicates that it also gives a relatively wide T<sub>m</sub> range of about 7 to 8 °C. 4-Bromopyrazole containing oligonucleotides also gave a relatively narrow melting range (3.6°C), but the average T<sub>m</sub> was significantly lower (42.8°C) than observed for nitropyrrole (44.7°C).

Table 3

<b>T<sub>m</sub> Data for Hybridization of the Sequence 5'-C<sub>2</sub>T<sub>5</sub>X T<sub>5</sub>G<sub>2</sub>-3' with 5'-G<sub>2</sub>A<sub>5</sub>Y A<sub>5</sub>C<sub>2</sub>-3'</b>	
<b>Base-pair (X-Y)</b>	<b>T<sub>m</sub></b>
C-G	58.6
C-A	43.2
C-T	48.7
A-G	50.1
A-C	44.9
A-T	57.4
Q <sub>2</sub> -G	43.2
Q <sub>2</sub> -C	45.1
Q <sub>2</sub> -A	45.9
Q <sub>2</sub> -T	44.6
B-G	42.5
B-C	41.4
B-A	45.0
B-T	42.4

Q<sub>2</sub> = 3-nitropyrrole deoxyribonucleoside, B = 4-bromopyrazole deoxyribonucleoside

The melting temperatures of the complementary oligonucleotide pairs were determined in 1.0 M NaCl, 0.1 mM EDTA, 10 mM sodium phosphate, pH 7.0 on a Jasco model 710 CD spectrometer equipped with a Peltier device for heating and cooling. The oligonucleotide concentration was 50 μM. The sample was placed in a 10 mm path length cuvette with a magnetic stir bar in the bottom, and the absorbance at 260 nm was monitored during the temperature cycle. The absorbance of the samples were measured at 2.5 or 5 °C intervals. The equilibration time at each temperature was determined by an initial test run employing a thermocouple placed into the cuvette.

These results with Q<sub>2</sub>, an analog designed to function as a universal nucleoside, have bearing on the convertide problem because they show that it is possible to design and construct a nucleoside analog that pairs with more than one natural nucleoside and is recognized by a DNA polymerase. Q<sub>2</sub> was functional in primer extension by T7 DNA polymerase even when located at the 3'-end of a primer. In comparison, mismatches at the 3'-end of the primer were not tolerated by T7 DNA polymerase. The experiments to determine which bases are preferentially written by Q<sub>2</sub> have not yet been done. If Q<sub>2</sub> were to "write" all four bases with equal frequency it would still be potentially useful as a convertide. As explained in the section "Rationale of Convertide Design" (first part of Section D., below) correct transpositions are propagated by the presence of primers terminated with a 3'-base complementary to the desired base. Incorrect transpositions would be recycled and read only by primers terminating in Q<sub>2</sub>.

Other preliminary studies that have been accomplished, include the synthesis of Q<sub>14</sub>, 2-carbamoyl-4-(2'-deoxy-β-D-ribofuranosyl)imidazole. This synthesis is described in a reprint included in the appendix[20]. Like Q<sub>2</sub>, Q<sub>14</sub> was designed to function as a universal nucleoside capable of pairing with all four natural bases (See Scheme 3). Q<sub>14</sub> has not yet been studied biochemically. In addition to these studies, syntheses of the known protected derivatives of Q<sub>5</sub> [21], Q<sub>9</sub> [8], Q<sub>12</sub> [4] and Q<sub>13</sub> [22] suitable for incorporation into oligonucleotides are underway. Brown has shown that primers containing Q<sub>12</sub> and Q<sub>13</sub> work well in PCR and that the *Taq* polymerase can extend off a primer having a 3'-Q<sub>12</sub>. [6]



**D. EXPERIMENTAL DESIGN**

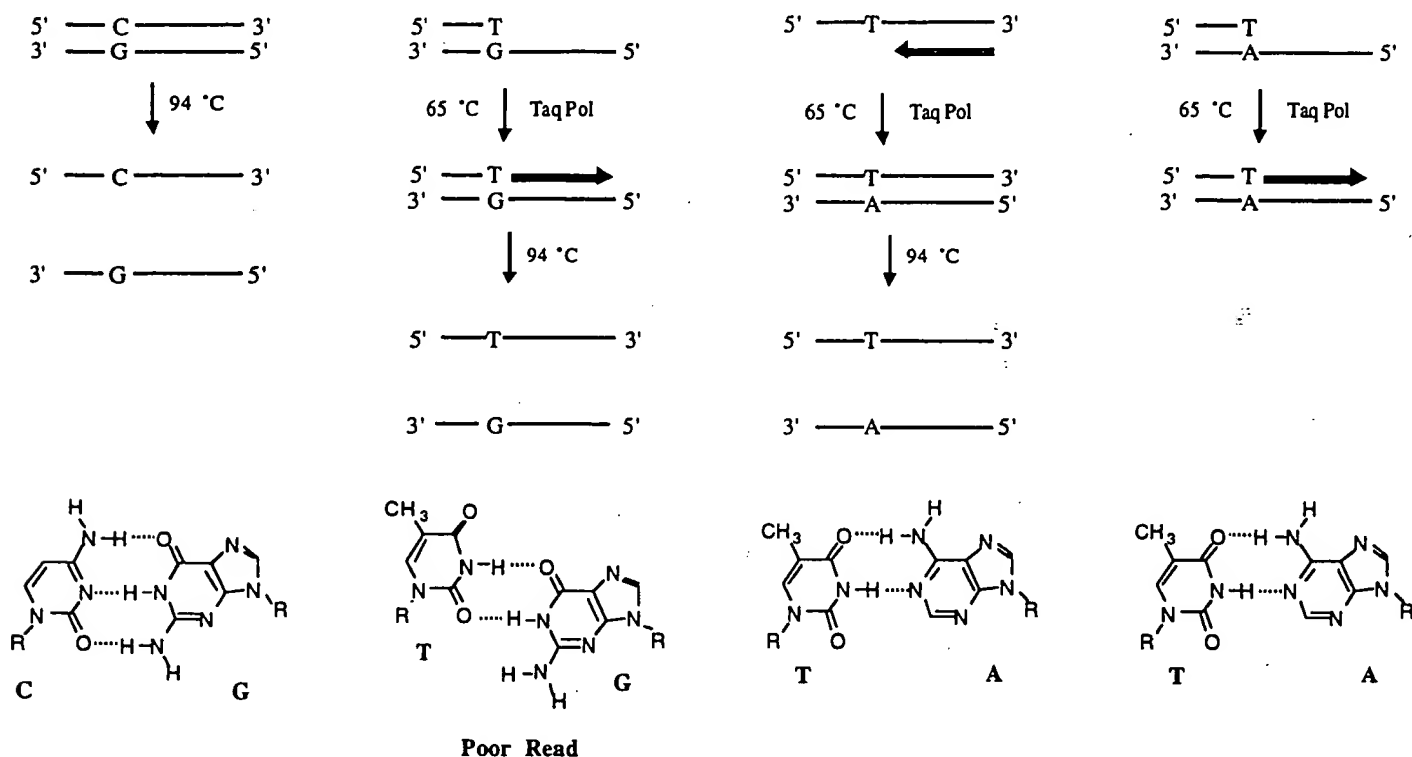
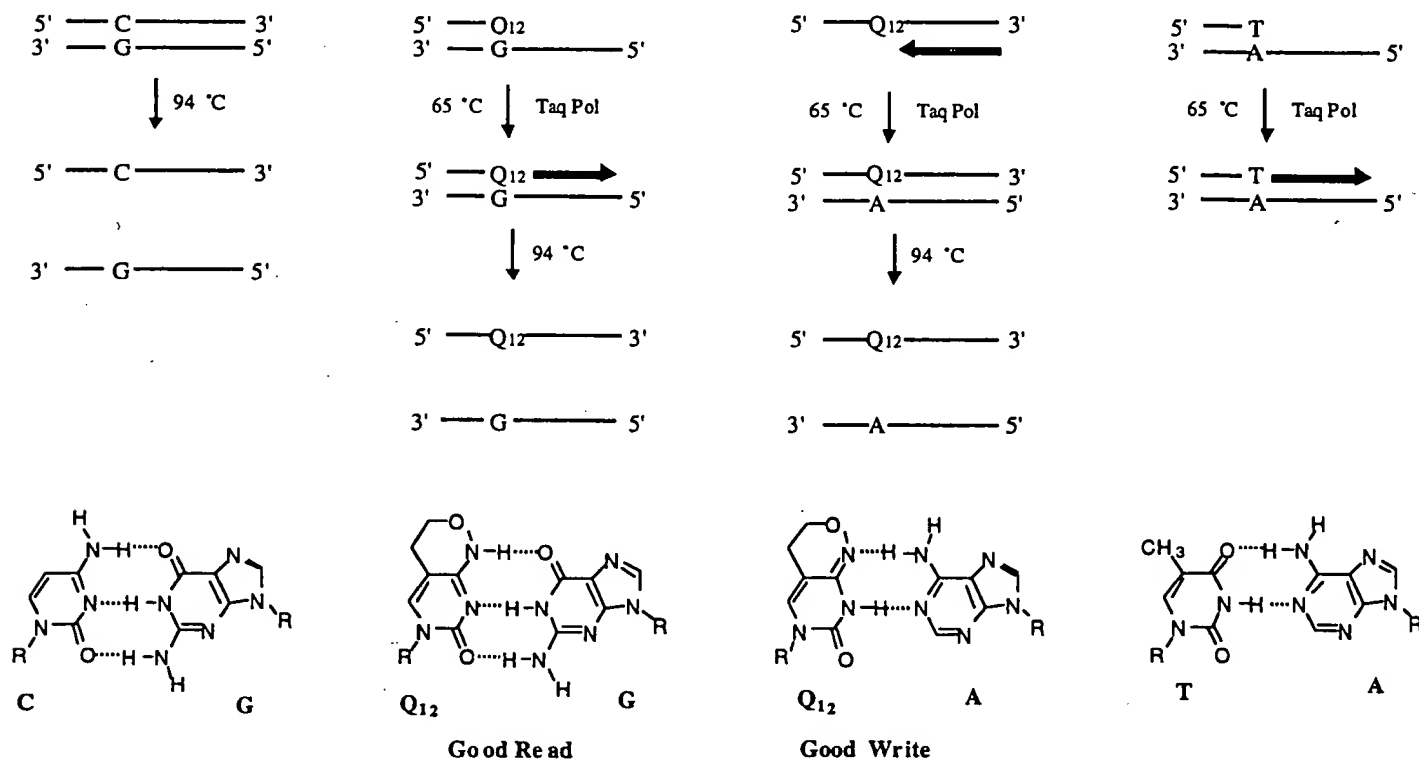
**Rationale of Convertide Design** – Conversion of a C-G base-pair to an A-T base-pair by use of a T-G wobble base-pair is illustrated in Scheme 2A. Application of convertide **Q<sub>12</sub>** to conversion of an C-G base-pair to an A-T base pair is compared in Scheme 2B. The latter should be more efficient because of the good "reading" and "writing" potential of **Q<sub>12</sub>** for G and A, respectively, resulting from its ability to tautomerize readily. A more detailed explanation of the biochemical experiments to determine the potential usefulness of each of the modified nucleosides is presented in the project 2 proposal. As illustrated in Scheme 2B for convertide **Q<sub>12</sub>**, the modified base is designed to provide a better base-pair with G than does T. Then, by virtue of a facile tautomeric shift to an alternate hydrogen bonding pattern, **Q<sub>12</sub>** can base-pair effectively with A, resulting in the conversion of G to an A. The reason that a variety of modified nucleosides, differing in geometry and potential for tautomeric shift and conformation change, need to be investigated, is because it is not possible to predict which of the unnatural base-pairs will be read and written most efficiently by the DNA polymerases.

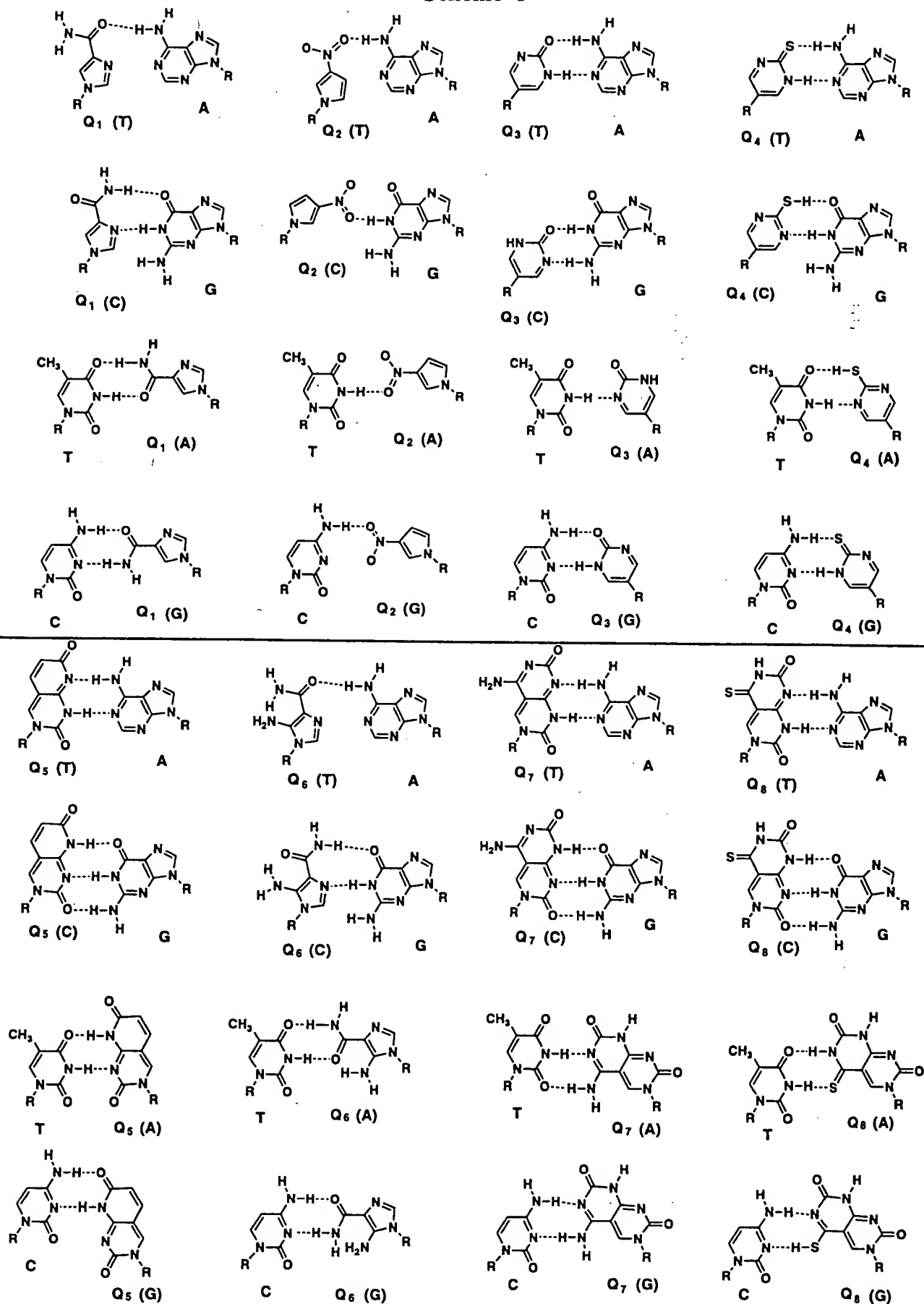
Scheme 3 shows the most likely hydrogen-bonding schemes for all the **Q**-bases with the natural bases. Table 4 summarizes the predicted functionality of each convertide in the proposed scheme of cancer detection. The main criteria were (1) the ability of the bases to obtain a particular tautomeric form (e.g., amino favored over imino for external nitrogen substituents) to pair with a complementary base and (2) minimization of distortion of the DNA duplex by keeping **Q**-N base-pairs iso-structural with the Watson Crick base-pairs so that a minimum of bulging or narrowing of the double helix is required.

**Table 4. Conversion of Natural Bases with Q-Bases**

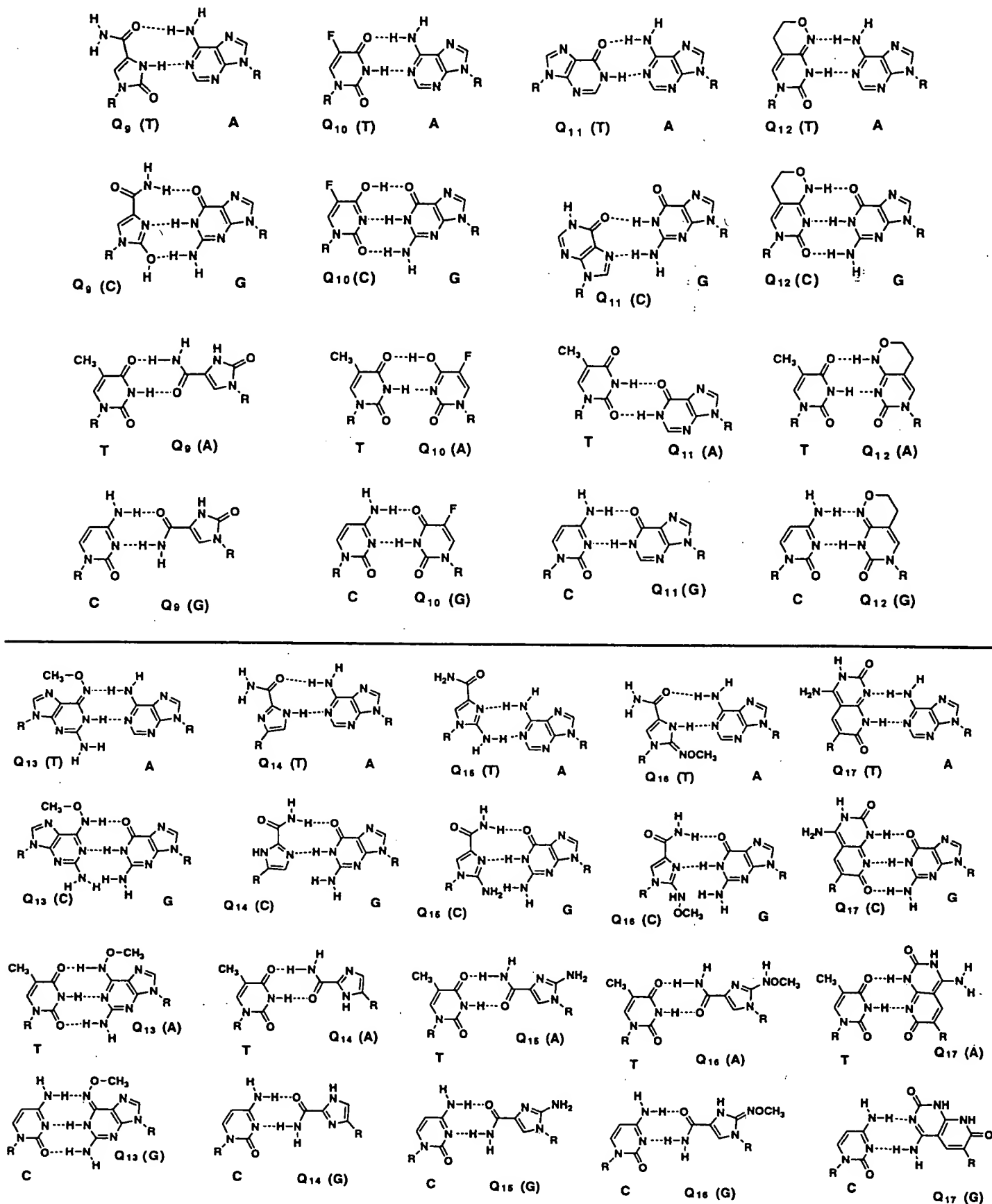
<b>Analog</b>	<b>Mimics</b>	<b>Writes Predicted base inserted by polymerase</b>	<b>Good Read Predicted <i>preferred</i> pairing to:</b>	<b>OK Read Predicted <i>possible</i> pairing to:</b>
<b>Q<sub>1</sub></b>	A, G, C	T, C, G	T, C, G	
<b>Q<sub>2</sub></b>	A, G, C, T	T, C, G, A	T, C, G, A	
<b>Q<sub>3</sub></b>	T	A	A, G	
<b>Q<sub>4</sub></b>	T, C	A, G	A, G	
<b>Q<sub>5</sub></b>	C, T	G, A	G, A	
<b>Q<sub>6</sub></b>	A, C, G?	T, G, C?	T, G	C?
<b>Q<sub>7</sub></b>	C, T, G	G, A, C	G, A, C	
<b>Q<sub>8</sub></b>	C, T, A, G	G, A, T, C	G, A, T, C	
<b>Q<sub>9</sub></b>	A, C, T	T, G, A	T, G, A	
<b>Q<sub>10</sub></b>	G	C	C, T	
<b>Q<sub>11</sub></b>	G	C	C, A	T, G
<b>Q<sub>12</sub></b>	T, C	A, G	A, G	
<b>Q<sub>13</sub></b>	A, G	T, C	C, T	A
<b>Q<sub>14</sub></b>	A, G, C, T	T, C, G, A	T, C, G, A	
<b>Q<sub>15</sub></b>	A, G	T, C	T, C	
<b>Q<sub>16</sub></b>	A, G, T	T, C, A	T, C, A	
<b>Q<sub>17</sub></b>	C, T, G	G, A, C	G, A,	

In some cases, more extensive modeling as a means to predict fit into a b-DNA helix was done. **Q<sub>12</sub>** and **Q<sub>14</sub>**, both designed to function as universal nucleosides, were inserted into helices generated in the program QUANTA and minimized by CHARMM. Both **Q<sub>12</sub>** and **Q<sub>14</sub>** fit opposite all four natural bases with only minor perturbation in the helix backbone. **Q<sub>2</sub>** has since been shown to be relatively non-discriminatory in base-pairing, as was discussed in section C above. In another example, **Q<sub>7</sub>** would have tautomeric forms

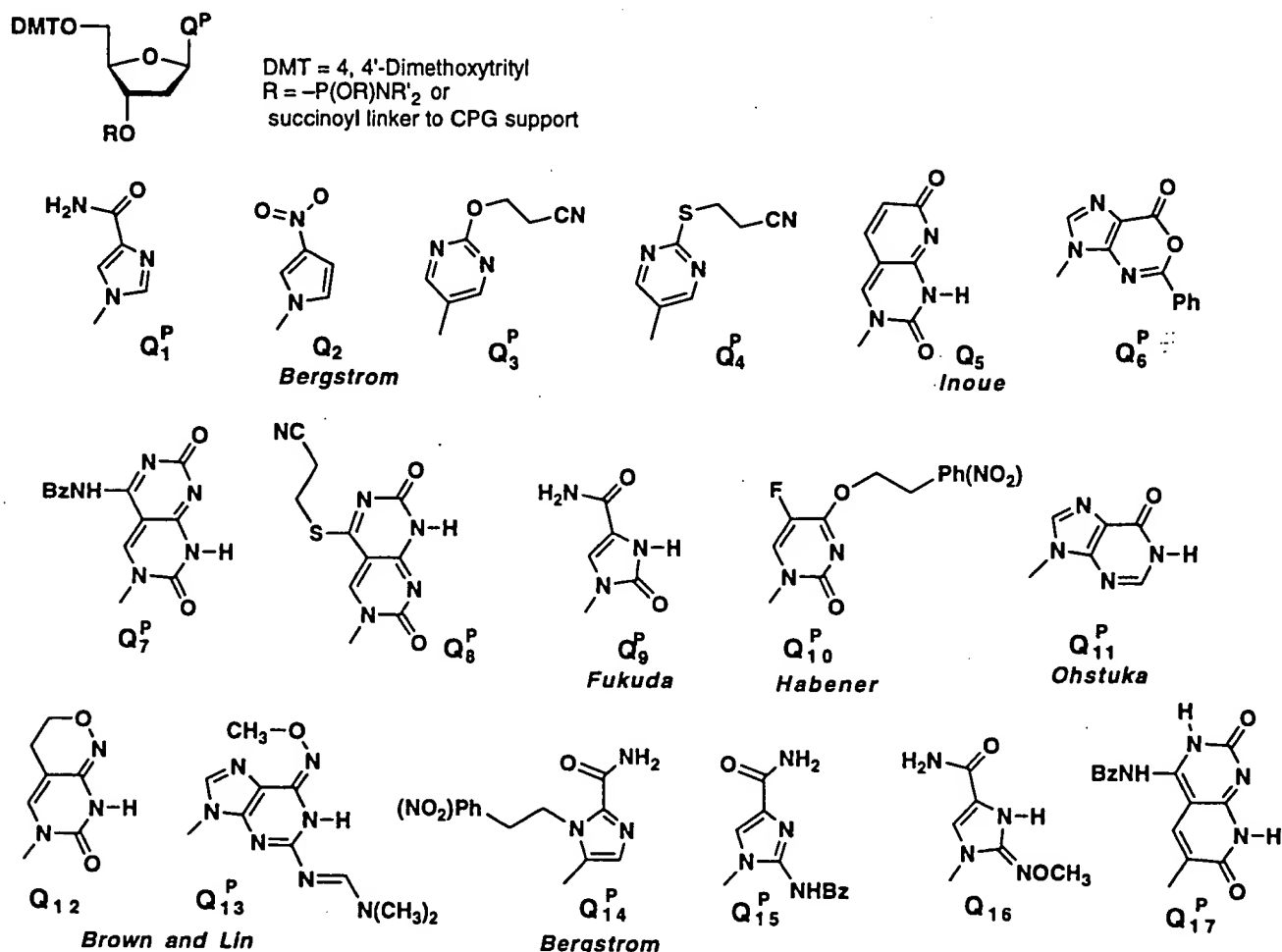
**Scheme 2 Conversion of C ≡ G to T = A****A.****B.**

**Scheme 3**

## Scheme 3 (cont)



## Scheme 4



For each previously synthesized protected mononucleoside, the name of the author of the key reference is denoted below. Structures which have no reference supplied are distinct to this proposal.

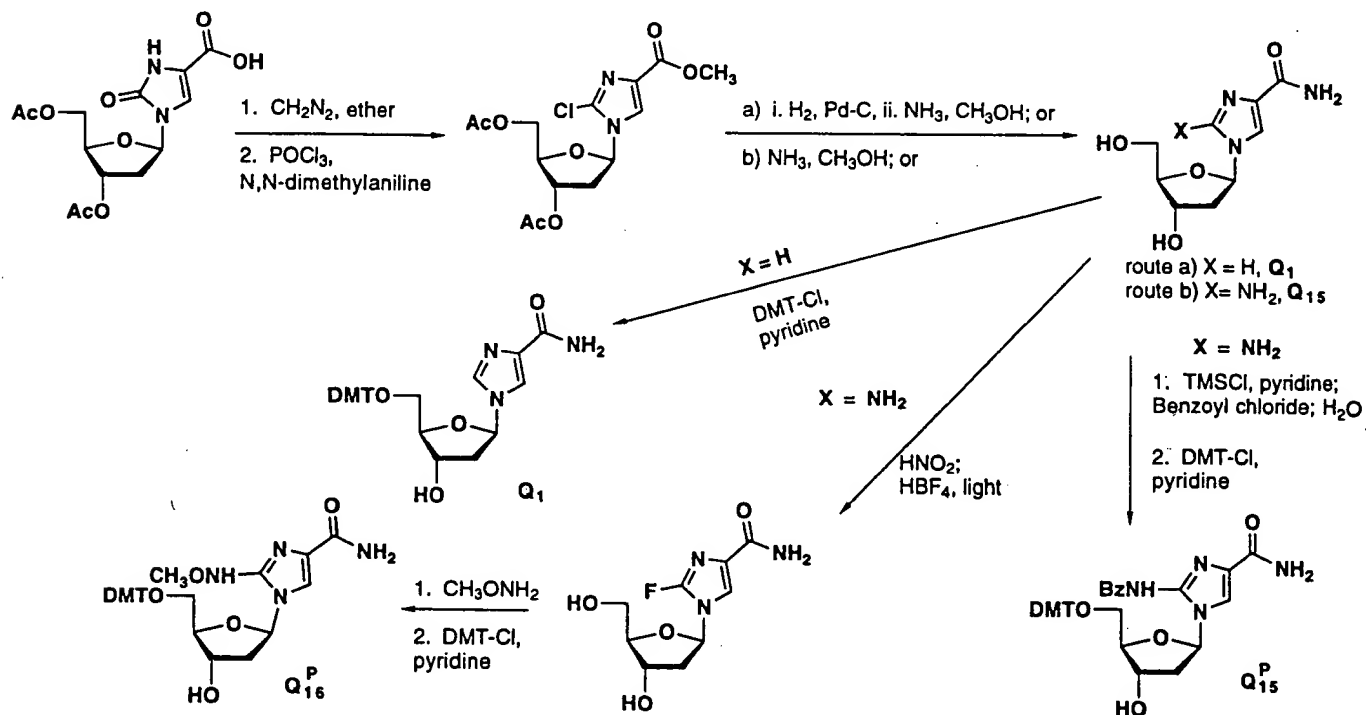
capable of hydrogen-bonding to all four of the bases, but when acting as an A-analog, the only hydrogen-bonding available forces **Q<sub>7</sub>** to be displaced towards the minor groove. Thus, **Q<sub>7</sub>** is a mimic for C, T, and G, but not A, and will potentially read and write the complementary bases G, A, and C, but not T. In reality, the function of convertides will be very enzyme-dependent and therefore these predictions will serve only as a guideline until PCR and ligation experiments can be carried out.

For all of this work, DMT-protected mononucleosides will be required for incorporation into oligonucleotides either at the 3'-terminus via attachment to a solid support or in the center of the chain using the 3'-phosphoramidite derivative. The structure of all these derivatives for convertides **Q<sub>1</sub>-Q<sub>17</sub>** are shown below.

The DMT and phosphoramidite derivatives of **Q<sub>10</sub>** and **Q<sub>11</sub>** are commercially available. Both **Q<sub>2</sub>** and **Q<sub>14</sub>** have been discussed in the Preliminary Results. The protected nucleosides of pyridopyrimidine **Q<sub>5</sub>** [21], carbamoyl-imidazolone **Q<sub>9</sub>** [8], the universal pyrimidine **Q<sub>12</sub>** [4], and universal purine **Q<sub>13</sub>** [22] will all be prepared according to the literature procedures.

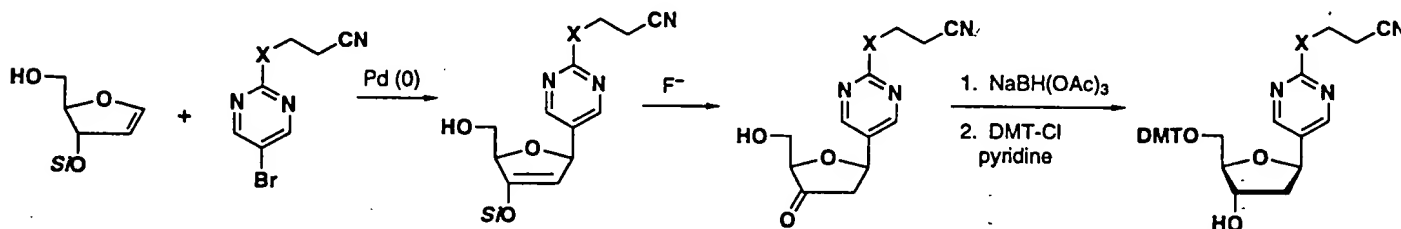
**Synthesis of Protected Convertides** – The protected derivatives of the N-1 linked 4-carboxamide imidazole nucleosides **Q<sub>1</sub>**, **Q<sub>15</sub>**, **Q<sub>16</sub>** will be prepared starting from the same precursor according to Scheme 4. The sugar protected **Q<sub>9</sub>** is synthesized according to the method of Fukuda [8] using chemistry developed by Otter *et al.* [23]. The free carboxyl group is first esterified with diazomethane and then reacted with phosphorus oxychloride in the presence of a base to convert the carbonyl function on the imidazole into a chloro-substituent. For the synthesis of **Q<sub>1</sub>** the chloro-imidazole bond is hydrogenolyzed and subsequent reaction with ammonia-saturated methanol converts the carboxylate ester into an amide and simultaneous cleaves the ester protecting groups on the sugar (see route (a) in Scheme 4 below). Alternatively, the direct reaction of the chloroimidazole nucleoside with ammonia-saturated methanol produces the 2-amino-4-carbamoyl nucleoside **Q<sub>15</sub>**, again with the ester protecting groups of the sugar removed (see route (b) in Scheme 5). Part of the synthesis of 4-carbamoyl-2-methoxyamino-imidazole deoxyribonucleoside **Q<sub>14</sub>** is patterned after that reported by Brown and Lin for N<sup>6</sup>-methoxy-2'-deoxyadenosine [22]. In their case, they were able to displace the chloro in 6-chloro-9-(2-deoxy-3,5-di-O-p-toluoyl-β-D-ribofuranosyl)purine by methoxyamine without cleaving the ester groups. That may be more difficult to accomplish with the imidazole. Thus following chemistry developed by Kirk *et al.* [24, 25], we have chosen to diazotize the 2-amino function of the imidazole and convert it the highly reactive 2-fluoro-imidazole, which can then be selectively displaced by methoxyamine without displacing any of the other ester groups. The desired 4-carbamoyl substituent is then generated by reaction with ammonia in methanol, which simultaneously cleaves the sugar ester groups, producing **Q<sub>16</sub>**. For all of these nucleosides, the 5'-dimethoxytrityl group is introduced in the standard way, except in the case of **Q<sub>15</sub>** which requires a preliminary protection of the 2-amino group as a benzamide.

Scheme 5

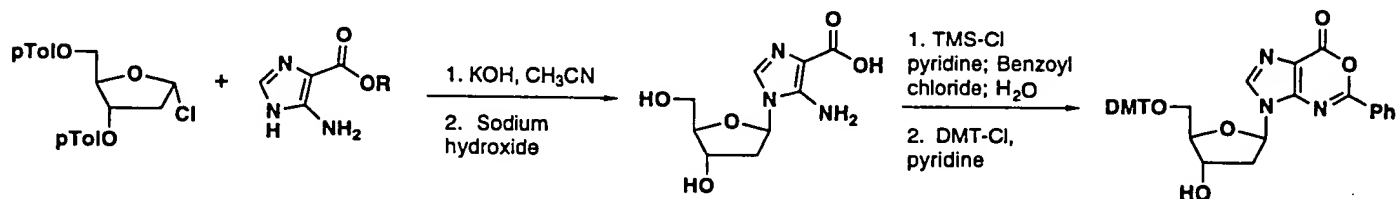


Protected forms of 5-(β-D-2'-deoxyribofuranosyl)-2-hydroxyl or -sulfhydryl)pyrimidine nucleotides (X = O or S; **Q<sub>3</sub>** and **Q<sub>4</sub>**) will be synthesized following a strategy developed by Daves and coworkers [26] for the preparation of C-nucleosides (see Scheme 6). The palladium(0)-mediated coupling of 3-*t*-butyldiphenylsilyl(*Si*)-protected glycal and cyanoethyl-protected pyrimidine (X = O or S) provides stereoselectively the β-C-(2,3-anhydro)-nucleoside. Removal of the silyl group with fluoride produces a 3'-ketonucleoside that can be stereoselectively reduced to provide the 2'-deoxyribonucleoside, which can be

readily 5'-protected with dimethoxytrityl(DMT) chloride to give the requisite nucleoside suitable for coupling to a solid support or preparation of a phosphoramidite. Note that the cyanoethyl group on the oxygen (or sulfur) atom (X) will be removed in the final deprotection step with concentrated aqueous ammonia to produce the 5-pyrimidinenucleoside with a free oxygen or sulfur atom at position 2.

**Scheme 6**

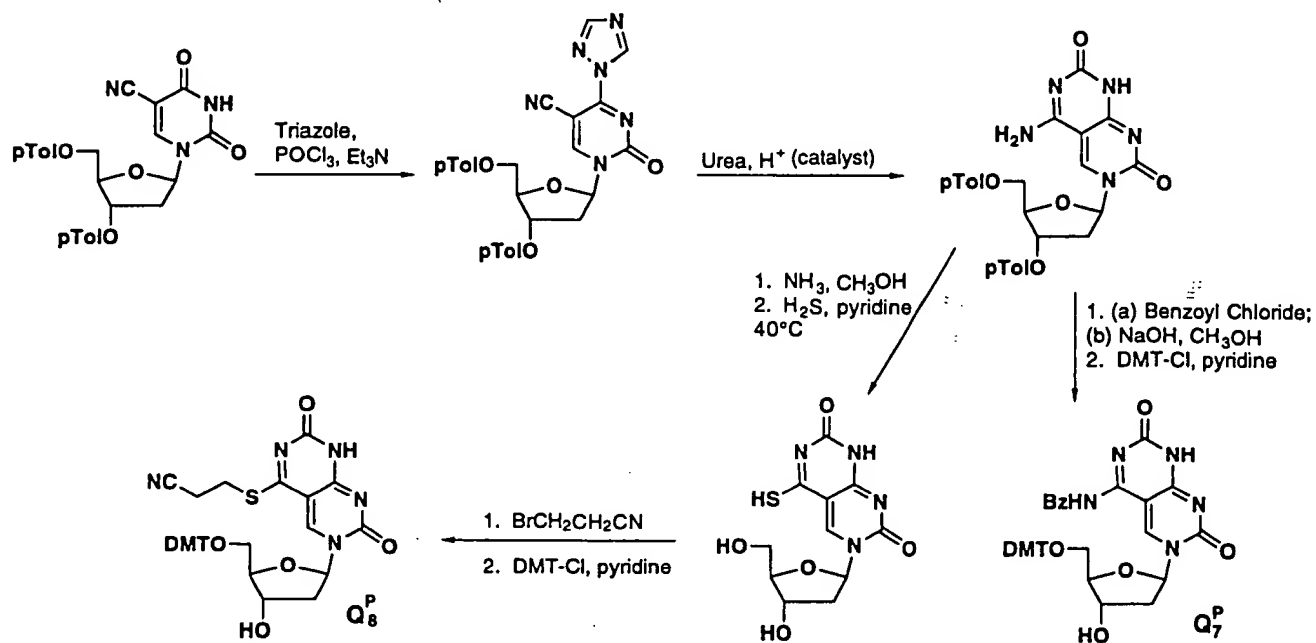
While 5-Amino-1-( $\beta$ -D-2'-deoxyribofuranosyl)imidazole-4-carboxamide (**Q<sub>6</sub>**) nucleotides have been accidentally incorporated into DNA by attempted chemical incorporation of an azidoimidazole nucleoside[27] and by enzymatic addition of **Q<sub>6</sub>** triphosphates [28], no general chemical method exists for controlled synthesis of **Q<sub>6</sub>**-containing oligonucleotides. The protected imidazole derivative prepared below (Scheme 7) would provide a general method for incorporating latent **Q<sub>6</sub>** nucleotides into synthetic oligonucleotides, which, at the end of the oligonucleotide synthesis, will generate the desired **Q<sub>6</sub>** residue by attack of ammonia onto the carbonyl of the imidoxazine heterocycle and subsequent ammonolysis of the benzamide.

**Scheme 7**

The reaction of the potassium salt of ethyl 5-aminoimidazole-4-carboxylate with 3,5-di-*p*-toluoyl-2-deoxy- $\alpha$ -D-ribofuranosyl chloride will produce the imidazole nucleoside [29], which is subsequently treated with sodium hydroxide to hydrolyze all of the ester bonds. Using the *in situ* silylation method [30], the free hydroxyl group will be blocked with trimethylsilyl groups and then subsequently the base portion reacted with benzoyl chloride, which reacts with the amino group and activates the carboxylate to produce the bicyclic imidoxazine nucleoside. Again the 5'-hydroxyl group will be protected as the DMT ether.

The pyrimidopyrimidines (**Q<sub>7</sub>**, **Q<sub>8</sub>**) will be prepared (Scheme 8) starting from a protected 5-cyano-2'-deoxyuridine, which will be made according to methods developed by Inoue and Ueda [31]. The 4-oxo substituent on the uracil ring is converted to a triazolidine that can then serve as a good leaving group. Under acid catalysis, urea will react at the 4-position and with the 5-cyano group to produce the second pyrimidine ring. This intermediate is easily converted to protected **Q<sub>7</sub>** by protection of the heterocyclic amine group, removal of the sugar ester groups, and protection of the 5'-hydroxyl as a DMT ether. The amino pyrimidopyrimidine can also be converted to **Q<sub>8</sub>**. After removal of the sugar ester groups, the amino-substituent is converted to a thiol group by reaction with hydrogen sulfide in pyridine [32,33]. For use in oligonucleotide synthesis, the thiol must be protected. This is easily accomplished by reaction with bromopropionitrile [34], after which the DMT-group can be introduced in the usual way.

## Scheme 8

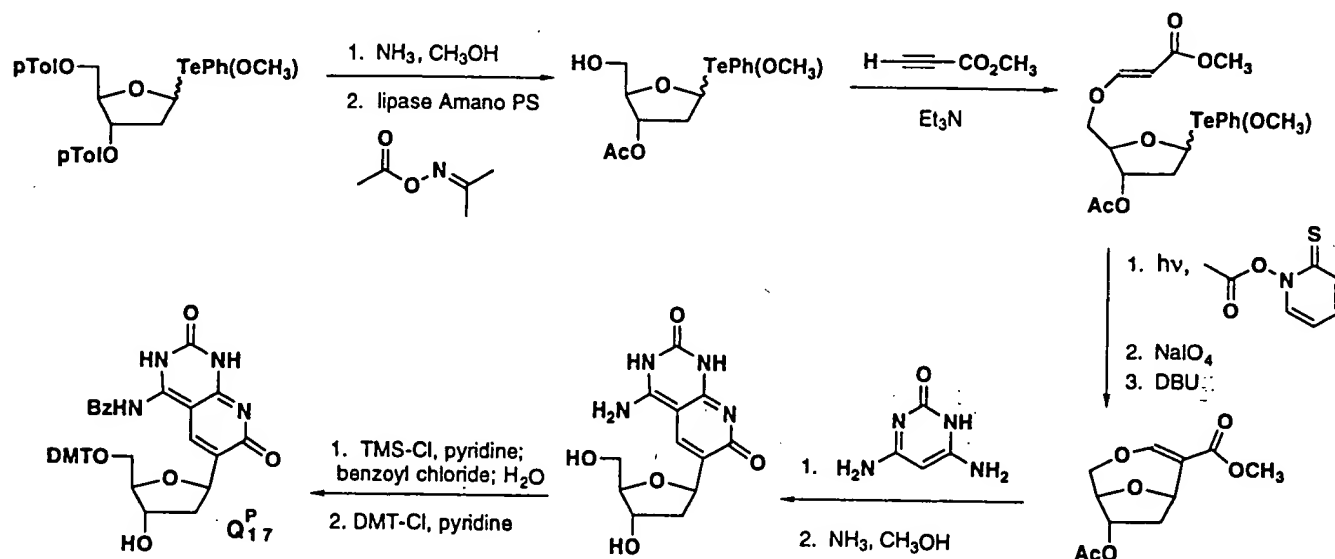


The proposed route for the preparation of pyridopyrimidine C-nucleoside **Q17** (Scheme 9) is closely modeled after the procedure developed by Barton and Ramesh for the synthesis of the C-nucleoside showdomycin, which took advantage of new free radical chemistry as a key step [35]. The starting anisoiltellerium ribose can be prepared by an analogous route used for a protected ribose by Barton [35]. The ester groups of the sugar are cleaved with ammonia in methanol and then lipase from *Amano PS* and an oxime ester are used to add an acetyl group selectively to the 3'-hydroxyl [36]. The 5'-hydroxyl can react with methyl propiolate in a Michael-type addition to position the acrylate acrylate. Photolysis of the *N*-acyloxy thiopyridone generates methyl radicals that homolytically cleave the tellerium anomeric linkage generating a C1-radical, which then should add selectively to the  $\alpha$ -carbon of the acrylate poised on the  $\beta$ -face of the sugar. The radical generated will then add to more thiopyridone to generate a  $\beta$ -thioester, which is oxidized and eliminated to regenerate the acrylate. Michael addition of diamino-pyrimidone (C-5) to the acrylate and subsequent *in situ* cyclization produces the desired free nucleoside **Q17**. Next, the hydroxy groups are transiently protected by trimethylsilylation [30] and the heterocyclic amino group is then benzoylated. The 5'-hydroxyl is converted to the DMT ether in the usual way.

**Oligonucleotide Synthesis, Purification and Characterization** – For preparation of primers containing internal base-modified nucleosides, the convertides will be incorporated as phosphoramidites using standard protocols or for preparation of oligomers with 3'-modified bases, **Q** nucleosides will be attached to a silica support via a succinate linker using recent improved methods [37]. This method is easy and fast to perform as no chromatographic purification of intermediates is required, uses only a two-fold excess of nucleoside succinate, and provides high nucleoside loading of the support ( $\sim 40 \mu\text{mol/g}$ ). The efficiency for each cycle of synthesis will be monitored by measuring the amount of the trityl blocking group released each cycle. Cleavage of the oligomers from the support and removal of the phosphate and bases protecting groups will be accomplished by reaction with concentrated ammonia, which in the case of **Q8** and **Q10** will be preceded by a DBU treatment to remove thiol protecting groups.



## Scheme 9



The synthetic oligonucleotides will be purified by ion-exchange HPLC or denaturing acrylamide gel electrophoresis followed by desalting steps. As it is essential to ensure that modified bases, particularly those that are potentially reagent sensitive are contained intact in the oligomers, the synthetic oligonucleotides will be further characterized by degradation to nucleosides with snake venom phosphodiesterase and alkaline phosphatase, and quantitation by HPLC [38].

If the efficiency of polymerization from a 3'-convertide is low, it may prove necessary to use other enzymes besides *Taq* polymerase. Some of these are "proofreading" enzymes that would most likely cleave off the Q-nucleotide, insert the natural nucleotide and then continue polymerization. To prevent this, the 5'-internucleotide linkage of the convertide will be a phosphorothioate instead of the natural phosphate. Phosphorothioate linkages are easily placed at any desired site in the phosphoramidite method by simple replacement of the normal oxidizing agent with a sulfurizing compound such as the Beaucage reagent [39] or tetraethylthiuram disulfide [40].

**Thermodynamic Stabilities of Oligomers Containing Convertides** – For those convertides which appear promising in initial biochemical studies, extensive melting temperature ( $T_m$ ) experiments will be performed, so that their effect on duplex stability can be determined. The ability to predict and fine-tune the duplex stability will be extremely important for optimizing both PCR as well as LDR. The  $T_m$ 's of self complementary oligonucleotides, which vary only in Q, the base pairing with Q, and the nearest neighbor bases, will be measured by recording the  $A_{260}$  of the oligonucleotide versus temperature. Thermodynamic data will be obtained by analyzing single melting curves according to the method of Petersheim and Turner [41] and this data will be compared to values obtained by measuring the concentration dependence of the  $T_m$  (van't Hoff analysis) for several oligonucleotides according to the method described by Marky and Breslauer [42].

### Convertides as Universal Nucleosides and as Intentional Mismatches

(Projects 1, 2, 4) – Nucleotide analogs will have two additional uses in cancer and disease detection. Incorporation of nucleotide analogues with less than optimal base-pairing near the discriminating base of an LDR primer may be used to enhance the specificity of cancer mutation detection (Project 4). In addition, nucleotide analogs may serve as universal base-pairs to help avoid problems in DNA detection caused by polymorphism surrounding the discriminating nucleotides (Projects 1, 2 and 4). Initially, analogue  $Q_2$  will be used in both these applications. As they become available, other Q-bases will be tested both as universal nucleosides and as

**Enhancement of the Hybridization Affinity of Oligonucleotides for Addressable Arrays (Project 5)** – For purposes of optimizing zip code capture, the  $T_m$  of the helix coil transition of DNA duplexes can be increased by incorporating certain modified bases in place of the natural bases. By replacing the natural bases at multiple sites in one or both strands, substantial increases in duplex thermal stability are possible. The analogue with thymine replaced by 5-propynyl-uracil can be used in either the complementary zip code (DNA or PNA) or zip code (DNA) or both. The  $T_m$  increases an average of 1.7 °C per substitution. [43] Thus, use of 5-propynyl-dU increases the stability of DNA/DNA and DNA/PNA hybrids and may also serve to narrow the  $T_m$  difference between all the zip code duplexes, which result from minor differences in GCcontent. Phosphoramidite derivatives of 5-propynyl-dU will be prepared according to Froehler. [43] The 5-propynyluracil PNA monomer with either Fmoc or Boc amino protection will be made following the published synthesis of PNA monomers [44,45] replacing thymine with 5-iodouracil and using Pd(0) coupling of the alkylated 5-iodouracil and propyne. See Project 5, Figure 4 for details.

## E. PROGRAM ASPECTS

The purpose of Project 3 is the design, synthesis of known and new nucleotide analogues, which will function as base-transposing agents or "convertides" in the cancer detection scheme. All of the 17 proposed convertides have ambiguous hydrogen bonding schemes which allow them to pair to one or more of the natural bases in a hybridization step ("read") and then subsequently function as an efficient template for another base in a polymerization reaction ("write"). A large part of the effort of the Bergstrom (Purdue University) and Hammer (Louisiana State University) teams will be spent on the chemical synthesis of the convertide mononucleosides, since only one known derivative (inosine,  $Q_{11}$ ) is commercially available. Convertides will be attached to solid support for incorporation at the 3'-terminus of oligonucleotides by Project 3 and evaluated for efficacy of base conversions in the cancer detection scheme by Core B.

The ultimate goal of this program project, to develop new methods for cancer detection, will pivot on the availability of oligonucleotides containing the 3'-convertides for the PCR/RE/LDR mutation detection scheme. In the design of these analogues there has already been considerable synergistic interactions between the members of Project 3 and Core B. As the convertide-containing primers become available from Project 3, they will be shipped to Core B for evaluation.

Successful convertide-based cancer detection will then be directly applicable to the clinical work of Project 1 (Lung and Colon Cancer) and Project 2 (Breast and Cervical Cancer). The new ability to detect low levels (1 in  $10^6$ ) of mutagenic genes by the PCR/RE/LDR detection scheme will allow unprecedented correlation of the presence of mutant sequences in a wide range of tissues to the clinical manifestation of these diseases.

Nucleotide analogs will have two additional uses in cancer and disease detection. Incorporation of nucleotide analogues near the discriminating base of an LDR primer may be used to enhance the specificity of cancer mutation detection (Project 4). In addition, nucleotide analogs may serve as universal base-pairs to help avoid problems in DNA detection caused by polymorphism surrounding the discriminating nucleotides (Projects 1, 2 and 4). Several of our collaborators, listed in Projects 1, 2, and 4 will benefit from nucleotide analogues. Please see letters of collaboration in the overview section of this program project grant.

In addition, Project 3 personnel will prepare both DNA and PNA 5-propynyluridine monomers for Project 5 for purposes of optimizing the  $T_m$  of the zip codes/complementary zip code duplexes.

Complete thermodynamic data will be measured on useful duplexes containing convertides. This information will be communicated to Core A (Informatics) for incorporation into computer programs for optimization of primer design in PCR/LDR, LDR/PCR, and PCR/RE/LCR protocols.

**F. TIMETABLE****Task 1. Syntheses of nucleoside convertides**

- a. Convertides Q<sub>2</sub>, Q<sub>5</sub>, Q<sub>6</sub>, Q<sub>9</sub>, Q<sub>10</sub>, Q<sub>12</sub>, and Q<sub>13</sub> will be prepared by literature methods or purchased. Months 1-24.
- b. Synthetic routes to the N-deoxynucleoside Q<sub>1</sub>, Q<sub>7</sub>, Q<sub>8</sub>, Q<sub>15</sub> and Q<sub>16</sub> will be developed as outlined in the proposal. Months 1-24.
- c. Synthetic routes to the C-deoxynucleoside Q<sub>3</sub>, Q<sub>4</sub>, Q<sub>14</sub>, and Q<sub>17</sub> will be developed as outlined in the proposal. Months 12-48.
- d. Convertides that prove promising on the basis of screening by core B and project 1 and 2 participants will be synthesized on larger scale. Months 24-60.

**Task 2. Syntheses of nucleoside convertide phosphoramidites and solid supports.**

- a. Dimethoxytrityl(DMT)-protected phosphoramidite derivatives of the convertides will be prepared. Months 6-60
- b. Long chain alkyl amine-controlled pore glass support (CPG) nucleoside linked convertides will be prepared. Months 6-60

**Task 3. Syntheses and testing of convertide modified oligonucleotides**

- a. Convertide phosphoramidites will be incorporated into oligonucleotides with sequences specified by core B and project 1 and 2 participants. The oligonucleotides will be submitted to core B for testing. Months 6-60.
- b. The convertide-CPG's will be used to incorporate the convertides into oligonucleotides at the 3'-end. The 3'-modified oligonucleotides will be submitted to core B for testing. Months 6-60.
- c. The thermodynamic stability of convertide modified oligonucleotides will be determined. Months 18-48

**G. HUMAN SUBJECTS / VERTEBRATE ANIMALS:** Not applicable

**I. CONSULTANTS/COLLABORATORS:** Letters and Biographical Sketches for collaborators are attached in the overview section of this program project grant.

**J. CONSORTIUM/CONTRACTUAL ARRANGEMENTS:** Please see following page.



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STATEMENT OF INTENT TO ESTABLISH A CONSORTIUM AGREEMENT

Date: January 26, 1994

Grant Number: P01-

P-01 Application Title: PROGRAM PROJECT: NEW METHODS FOR  
CANCER DETECTION

Project # 3; DESIGN AND SYNTHESIS OF  
NUCLEOTIDE ANALOGUES.

Proposed Project Period: Year 01; 12/01/94- 11/30/95

"The appropriate programmatic and administrative personnel of each institution involved in this grant application are aware of the NIH consortium grant policy and will establish the necessary inter-institutional agreement(s) consistent with that policy."

CORNELL UNIVERSITY MEDICAL  
COLLEGE, NEW YORK, NY

LOUISIANA STATE UNIV.  
BATON ROUGE, LA.

(Applicant Institution)

(Consortium Institution)

\_\_\_\_\_  
(name) (date)  
Principal Investigator:

FRANCIS BARANY, Ph.D.

\_\_\_\_\_  
(name) (date)  
Co-Investigator:

ROBERT HAMMER, Ph.D.

\_\_\_\_\_  
(name) (date)  
Official Authorized to Sign for Institution

GREGORY W. SISKIND, M.D.  
ASSOCIATE DEAN

\_\_\_\_\_  
(name) (date)  
Official Authorized to Sign for Institution

CORNELL UNIVERSITY MEDICAL COLLEGE  
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STATEMENT OF INTENT TO ESTABLISH A CONSORTIUM AGREEMENT

**Date:** January 26, 1994

**Grant Number:** P01-

**P-01 Application Title:** PROGRAM PROJECT: NEW METHODS FOR  
CANCER DETECTION

Project # 3; DESIGN AND SYNTHESIS OF  
NUCLEOTIDE ANALOGUES.

**Proposed Project Period:** Year 01; 12/01/94- 11/30/95

"The appropriate programmatic and administrative personnel of each institution involved in this grant application are aware of the NIH consortium grant policy and will establish the necessary inter-institutional agreement(s) consistent with that policy."

CORNELL UNIVERSITY MEDICAL  
COLLEGE, NEW YORK, NY

(Applicant Institution)

PURDUE UNIVERSITY  
WEST LAFAYETTE, IN.

(Consortium Institution)

\_\_\_\_\_  
(name) (date)  
Principal Investigator:

FRANCIS BARANY, Ph.D.

\_\_\_\_\_  
(name) (date)  
Co-Investigator:

DONALD BERGSTROM, Ph.D.

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GREGORY W. SISKIND, M.D.  
ASSOCIATE DEAN

\_\_\_\_\_  
(name) (date)  
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**K. LITERATURE CITED.**

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## **Project 4.**

### **Engineering an Improved Thermostable Ligase**

**Project Leader: Francis Barany  
Cornell University Medical College**

**Project Co-Leader: Aneel Aggarwal  
College of Physicians & Surgeons of Columbia University**

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. DO NOT EXCEED THE SPACE PROVIDED.

One of the fundamental problems in detecting cancers in tissue samples is the need to distinguish a few cells containing the cancer mutation from the vast majority of normal cells. We have developed a novel polymerase chain reaction/ligase detection reaction method (PCR/LDR) for high throughput, low sensitivity mutation detection (1 in  $10^2$  to  $10^3$ ), and a PCR/restriction endonuclease/LDR (PCR/RE/LDR) method for high sensitivity mutation detection (1 in  $10^6$  to 1 in  $10^7$ , see Project 1 and 2). The enzyme which provides the specificity for these methods is *Tth* ligase, the gene for which was originally cloned in our laboratory. The limit of detection of these two methods would be significantly improved by increasing the specificity of *Tth* ligase.

We are developing a comprehensive approach to understanding the mechanism of *Tth* ligase action, and improving its fidelity for discriminating perfectly matched from mismatched substrates. The three parts to this program are: (i) Developing a rapid assay to test different reaction conditions, mutant *Tth* ligases, and modified DNA substrates for their effect on the fidelity of ligase. This assay has already shown that *Tth* ligase demonstrates higher sensitivity when the discriminating base is on the 3' end of the test primer. Introducing a nucleotide analogue (see Project 3) or mismatched base adjacent to or near the discriminating base may increase the specificity of this reaction. Such modified oligonucleotide primers will be tested in our fidelity assay using wild type and mutant *Tth* ligase. (ii) Determining the 3-dimensional structure of *Tth* ligase-DNA complex. This structure will help to reveal the mechanism of DNA ligation, and provide an understanding of the specificity of the enzyme for mismatches at the nicked site. (iii) Using site-specific mutagenesis to construct mutant *Tth* ligases. Design of these mutants will be based on protein sequence homology and protein-sugar-phosphate backbone contacts as determined from the X-ray structure. We have already isolated and partially characterized over 30 site-specific *Tth* ligase mutants, and these will be tested in our fidelity assay.

PERSONNEL ENGAGED ON PROJECT, INCLUDING CONSULTANTS/COLLABORATORS. Use continuation pages as needed to provide the required information in the format shown below on *all* individuals participating in the project.

Name	<u>BARANY, Francis</u>	Degree(s)	<u>Ph.D.</u>	Social Security #	<u>REDACTED</u>
Position Title	<u>Associate Professor</u>	D.O.B.	<u>REDACTED</u>	Role on Project	<u>Prin. Investig.</u>
Organization	<u>Cornell University Medical College</u>			Department	<u>Microbiology</u>
Name	<u>AGGARWAL, Aneel</u>	Degree(s)	<u>Ph.D.</u>	Social Security #	<u>REDACTED</u>
Position Title	<u>Assistant Professor</u>	D.O.B.	<u>REDACTED</u>	Role on Project	<u>Co-investigator</u>
Organization	<u>College of Physicians &amp; Surgeons of Columbia University</u>			Department	<u>Biochem &amp; Biophys</u>
Name	<u>LUO, Jianying</u>	Degree(s)	<u>Ph.D.</u>	Social Security #	<u>REDACTED</u>
Position Title	<u>Research Associate</u>	D.O.B.	<u>REDACTED</u>	Role on Project	<u>REDACTED</u>
Organization	<u>Cornell University Medical College</u>			Department	<u>Microbiology</u>
Name		Degree(s)		Social Security #	
Position Title		D.O.B.		Role on Project	
Organization				Department	
Name		Degree(s)		Social Security #	
Position Title		D.O.B.		Role on Project	
Organization				Department	
Name		Degree(s)		Social Security #	
Position Title		D.O.B.		Role on Project	
Organization				Department	
Name		Degree(s)		Social Security #	
Position Title		D.O.B.		Role on Project	
Organization				Department	

## FROM

FROM

THROUGH

94/12/01

95/11/30

**\$52,347**

**BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD  
DIRECT COSTS ONLY**

PROJECT 4

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED			
			2nd	3rd	4th	5th
<b>PERSONNEL:</b>						
<i>Salary &amp; fringe benefits</i>						
<i>Applicant organization only</i>		\$37,847	\$39,361	\$40,935	\$42,572	\$44,275
<b>CONSULTANT COSTS</b>		\$0	\$0	\$0	\$0	\$0
<b>EQUIPMENT</b>		\$0	\$2,000	\$2,000	\$2,000	\$2,000
<b>SUPPLIES</b>		\$10,500	\$10,920	\$11,357	\$11,811	\$12,283
<b>TRAVEL</b>		\$1,000	\$1,040	\$1,082	\$1,125	\$1,170
<b>PATIENT CARE COSTS</b>	<b>INPATIENT</b>	\$0	\$0	\$0	\$0	\$0
	<b>OUTPATIENT</b>	\$0	\$0	\$0	\$0	\$0
<b>ALTERATIONS AND RENOVATIONS</b>		\$0	\$0	\$0	\$0	\$0
<b>OTHER EXPENSES</b>		\$3,000	\$3,120	\$3,245	\$3,375	\$3,510
<b>SUBTOTAL DIRECT COSTS</b>		\$52,347	\$56,441	\$58,619	\$60,883	\$63,238
<b>CONSORTIUM/ CONTRACTUAL COSTS</b>		\$0	\$0	\$0	\$0	\$0
<b>TOTAL DIRECT COSTS</b>		\$52,347	\$56,441	\$58,619	\$60,883	\$63,238
<b>TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD</b>						<b>\$291,528</b>

(Item 8a)-&gt;

**JUSTIFICATION** (Use continuation pages if necessary):

**From Budget for Initial Period:** Describe the specific functions of the personnel, collaborators, and consultants and identify individuals with appointments that are less than full time for a specific period of the year, including VA appointments.

**For All Years:** Explain and justify purchase of major equipment, unusual supplies requests, patient care costs, alterations and renovations, tuition remission, and donor/volunteer costs.

**From Budget for Entire Period:** Identify with an asterisk (\*) on this page and justify any significant increase or decrease in any category over the initial budget period. Describe any change in effort of personnel.

**For Competing Continuation Applications:** Justify any significant increases or decreases in any category over the current level of support.

**Personnel:** Cornell University Medical College salaries are in accordance with the high cost of living in New York City, as well as the experience of the personnel. A 10% effort by the Principal Investigator full supervision of the junior personnel in this project. Cornell University Medical College has granted the Principal Investigator a Hirsch/Monique Weill-Caulier Career Scientist Award from 01/01/92 to 01/01/1997. This award of \$20,000 / year may be used as salary (and fringe benefit support) only. It thus allows the P.I. to spend full effort on research.

Dr. Jianying Luo is a research associate (Ph.D.) who has been in the Principal Investigator's laboratory since 9/01/92. Dr. Luo obtained her Ph.D. in the laboratory of Dr. Joseph Krakow at Hunter College. She is a highly skilled member of the Principal Investigator's laboratory. She has constructed, isolated, sequenced, and characterized the proteins from over 30 site-specific mutants of the *Tth* ligase gene.

In addition, she has performed all the ligase fidelity assays described in the preliminary results. The high cost of living in New York City necessitates competitive salaries.

*Fringe benefits:* Cornell University Medical College fringe benefits from 12/1/94 to 11/30/95 are at 32%. Salary increases of 4% are in accordance with Cornell University Medical College guidelines. Cornell University Medical College documentation of calculation of the indirect cost and fringe rate are attached.

*Equipment:* Our equipment situation is reasonably good, as most of our equipment was bought 8 years ago. We are continuously looking for ways to salvage equipment and cut costs. Due to continuing budget cuts, Dr. William Holloman (Professor at Cornell, shares laboratory space with the Principal Investigator) and the P.I. have made a concerted effort to pool our resources and share as much equipment as possible. This occasionally means re-arranging our experiments while waiting several days for the FPLC to be free, but it has worked out quite well and strengthened the interactions between our two laboratories. When I had the only thermal cycler, both labs co-ordinated its use without difficulty. We have shared in the expense of shared computers, computer programs such as DNA-Star, gel dryers, vacuum pumps, and service contracts. No equipment costs are requested for this grant for the first year. Replacement of used equipment and ability to buy smaller items is requested for years 2-5.

Our capacity to synthesize large numbers of oligonucleotides, perform large numbers of LCR, LDR, and PCR amplifications, and fluorescent quantification of LDR products on a DNA sequencer has been tremendously augmented by generous equipment gifts from Roche Molecular Diagnostics, Perkin Elmer, and Applied Biosystems Inc. Specifically, we now have our own PE 9600 thermal cycler, ABI 394 DNA synthesizer, and an ABI 373 automated DNA sequencer. Although these instruments were placed in my laboratory for use in developing the Ligase Chain Reaction (LCR) for detection of infectious and genetic diseases and isolating new thermophilic proteins, all three companies have given their encouragement to use these instruments for cancer research. Such equipment still necessitates highly skilled and motivated personnel to run them. See Core B.

*Supplies.* We are perpetually underfunded in these categories, but the submitted budget is in compliance with our past levels of support. Our DNA modifying enzymes include restriction enzymes (we make our own *TaqI*), T4 kinase, T4 Ligase, Klenow, *Taq* Polymerase, and other enzymes. Chemicals and reagents include all our chemicals, column matrixes, miniprep columns, as well as chemicals required for oligonucleotide synthesis. Other supplies include radioisotopes, media, and film.

*Travel:* We request support for the Principal Investigator to attend one domestic meeting per year in order to present results and learn of advances related to protein engineering and DNA interacting proteins as described in this proposal.

*Publication and report costs.* We request support for phone, Fax, printing, and photocopying of \$3,000 for the first year.

**DETAILED BUDGET FOR INITIAL BUDGET PERIOD**

FROM

THROUGH

94/12/01

95/11/30

### DIRECT COSTS ONLY

PERSONNEL (Applicant Organization Only)					DOLLAR AMOUNT REQUESTED (omit cents)		
NAME	ROLE ON PROJECT	TYPE APPT. (months)	% EFFORT ON PROJ.	INST. BASE SALARY	SALARY REQUESTED	FRINGE BENEFITS	TOTALS
Aneel K. Aggarwal	Principal Investigator	12	10				
To be appointed	Post-Doc Fellow	12	100				
SUBTOTALS					\$34,670	\$11,615	\$46,285
CONSULTANT COSTS							\$0
EQUIPMENT (Itemize)							\$0
SUPPLIES (Itemize by category)							\$0
Chemicals & Reagents \$3,500							
X-ray supplies \$5,000							
Plastic and Glassware \$2,000							
							\$10,500
TRAVEL							
Synchrotron visits \$1,500							\$1,500
PATIENT CARE COSTS		INPATIENT					\$0
		OUTPATIENT					\$0
ALTERATIONS AND RENOVATIONS (Itemize by category)							\$0
OTHER EXPENSES (Itemize by category)							
Phone, Xerox, Page Char; \$3,000							\$3,000
SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD							\$61,285
CONSORTIUM/CONTRACTUAL COSTS							
DIRECT COSTS					TOTAL		\$40,448
INDIRECT COSTS 66%							
TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD (Item 7a, Face Page)							\$101,733

**BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD  
DIRECT COSTS ONLY**

PROJECT 4

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED			
			2nd	3rd	4th	5th
PERSONNEL:						
Salary & fringe benefits						
Applicant organization only		\$46,285	\$48,136	\$50,061	\$52,063	\$54,146
CONSULTANT COSTS		\$0	\$0	\$0	\$0	\$0
EQUIPMENT		\$0	\$0	\$0	\$0	\$0
SUPPLIES		\$10,500	\$10,920	\$11,357	\$11,811	\$12,283
TRAVEL		\$1,500	\$1,560	\$1,622	\$1,687	\$1,754
PATIENT CARE COSTS	INPATIENT	\$0	\$0	\$0	\$0	\$0
	OUTPATIENT	\$0	\$0	\$0	\$0	\$0
ALTERATIONS AND RENOVATIONS		\$0	\$0	\$0	\$0	\$0
OTHER EXPENSES		\$3,000	\$3,120	\$3,245	\$3,375	\$3,510
SUBTOTAL DIRECT COSTS		\$61,285	\$63,736	\$66,285	\$68,936	\$71,693
CONSORTIUM/ CONTRACTUAL COSTS		\$40,448	\$42,066	\$43,749	\$45,499	\$47,319
TOTAL DIRECT COSTS		\$101,733	\$105,802	\$110,034	\$114,435	\$119,012
TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD (Item 8a)->						<b>\$551,016</b>

## JUSTIFICATION (Use continuation pages if necessary):

**From Budget for Initial Period:** Describe the specific functions of the personnel, collaborators, and consultants and identify individuals with appointments that are less than full time for a specific period of the year, including VA appointments.

**For All Years:** Explain and justify purchase of major equipment, unusual supplies requests, patient care costs, alterations and renovations, tuition remission, and donor/volunteer costs.

**From Budget for Entire Period:** Identify with an asterisk (\*) on this page and justify any significant increase or decrease in any category over the initial budget period. Describe any change in effort of personnel.

**For Competing Continuation Applications:** Justify any significant increases or decreases in any category over the current level of support.

**Personnel:** The funds requested will cover 10% of the annual salary of Dr. Aneel Aggarwal. Dr. Aggarwal is a co-investigator of this project, and the structure determination of Taq ligase will be performed in his laboratory. He will guide a postdoctoral fellow as to the various aspects of structure determination, and will lend general assistance with data collection, data processing, program implementation etc.

Dr. Aggarwal will recruit a postdoctoral fellow to undertake the structure determination of Taq ligase with and without DNA. The postdoctoral fellow will also undertake a biochemical study to determine the factors affecting complex formation between Taq ligase and DNA. Determination of these factors is important in identifying the optimal conditions for crystal growth. This is a full-time position and a candidate with a background in physics and biochemistry will be sought.

*Travel:* The funds requested are to help cover part of the cost of collecting X-ray data at a synchrotron facility. Synchrotron radiation combined with image plates yields the best data attainable from any given crystal. Synchrotron radiation can also in some cases help extend the diffraction limit of crystals.

*Supplies:* The money requested is to cover the cost of basic supplies for crystallization of proteins and nucleic acids. Chemical supplies include crystallization reagents such as polyethylene glycol, ammonium sulfate etc., and oligonucleotides. X-ray supplies are X-ray films, developer and fixer, goniometers, wrenches, quartz and glass capillaries for mounting crystals, lead gloves, scintillating crystals etc. Glassware and plasticware funds are for an assortment of beaker, measuring cylinders, flasks, tissue culture trays for crystallization, disposable filter systems, disposable pipettes etc.



**BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD  
DIRECT COSTS ONLY**

PROJECT 4

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED			
			2nd	3rd	4th	5th
<b>PERSONNEL:</b>						
<i>Salary &amp; fringe benefits</i>						
<i>Applicant organization only</i>		\$84,132	\$87,497	\$90,997	\$94,635	\$98,420
<b>CONSULTANT COSTS</b>		\$0	\$0	\$0	\$0	\$0
<b>EQUIPMENT</b>		\$0	\$2,000	\$2,000	\$2,000	\$2,000
<b>SUPPLIES</b>		\$21,000	\$21,840	\$22,714	\$23,623	\$24,568
<b>TRAVEL</b>		\$2,500	\$2,600	\$2,704	\$2,812	\$2,924
<b>PATIENT CARE COSTS</b>	<b>INPATIENT</b>	\$0	\$0	\$0	\$0	\$0
	<b>OUTPATIENT</b>	\$0	\$0	\$0	\$0	\$0
<b>ALTERATIONS AND RENOVATIONS</b>		\$0	\$0	\$0	\$0	\$0
<b>OTHER EXPENSES</b>		\$6,000	\$6,240	\$6,490	\$6,750	\$7,020
<b>SUBTOTAL DIRECT COSTS</b>		\$113,632	\$120,177	\$124,904	\$129,819	\$134,931
<b>CONSORTIUM/ CONTRACTUAL COSTS</b>		\$40,448	\$42,066	\$43,749	\$45,499	\$47,319
<b>TOTAL DIRECT COSTS</b>		\$154,080	\$162,243	\$168,653	\$175,318	\$182,250
<b>TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD</b>						<b>\$842,544</b>
						<i>(Item 8a)-&gt;</i>

**JUSTIFICATION (Use continuation pages if necessary):**

**From Budget for Initial Period:** Describe the specific functions of the personnel, collaborators, and consultants and identify individuals with appointments that are less than full time for a specific period of the year, including VA appointments.

**For All Years:** Explain and justify purchase of major equipment, unusual supplies requests, patient care costs, alterations and renovations, tuition remission, and donor/volunteer costs.

**From Budget for Entire Period:** Identify with an asterisk (\*) on this page and justify any significant increase or decrease in any category over the initial budget period. Describe any change in effort of personnel.

**For Competing Continuation Applications:** Justify any significant increases or decreases in any category over the current level of support.

**Total 5yr budgets for Project 4**

## RESOURCES AND ENVIRONMENT

FACILITIES: Mark the facilities to be used at each performance site listed in Item 9, Face Page, and briefly indicate their capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Use "Other" to describe the facilities at any other performance sites listed in Item 9 on the Face Page and at sites for field studies. Use continuation pages if necessary. Include an explanation of any consortium/contractual arrangements with other organizations.

☒ Laboratory: The Barany group currently numbers 6 full time researchers including the P.I., and one part time technician. The lab is on the fourth floor of the microbiology wing, and comprises 3,500 sq. ft. of relatively new space (8 yrs since renovation.) The Barany lab is 670 sq. ft., in addition, a cold room, an equipment room, a dark room and a computer room are shared with Dr. William Holloman.

☐ Clinical:

☐ Animal:

☒ Computer: The P.I. has a Macintosh Quadra 840 AV (in his office), four Macintosh IIfx, and one Macintosh Classic computer which is used primarily to program our HPLC. Our Microbiology Dept. has a SPARCstation 2 with the Genetics Computer Group package of programs for retrieval and analysis of protein and DNA sequences. We are also directly connected with the Rockefeller University computer.

☒ Office: The P.I. has a private office of about 180 sq. feet.

☐ Other ( ): \_\_\_\_\_

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

With Dr. Holloman, the P.I. share one ultracentrifuge, 2 high speed and 2 low speed centrifuges, several microfuges, a liquid scintillation counter, 2 Vis/UV spectrophotometers (one with kinetics), a fluorescence spectrophotometer, 2 PE thermal cyclers, an FPLC, an HPLC, 3 ultralow freezers, a fermenter, 2 floor shakers, 2 chemical hoods, a biological hood, a french press, a sonicator, a lyophilizer, a speed vac, a rotary evaporator, several water bath shakers, fraction collectors, gel dryers, power supplies, freezers, refrigerators, and incubators. Our department has a phosphorimager which is shared among our faculty. In addition, Roche, PE, and ABI, have placed a PE 9600 thermal cycler, an ABI 394 DNA synthesizer, and an ABI 373 automated DNA sequencer in my laboratory with encouragement to use these instruments for research on the Cancer detection studies, as well as for the other projects.

ADDITIONAL INFORMATION: Provide any other information describing the environment for the project. Identify support services such as consultant, secretarial, machine shop, and electronics shop, and the extent to which they will be available to the project.

Our department of Microbiology has grown to 7 members under the leadership of Dr. Kenneth I. Berns. He has forged a joint Graduate program in Molecular Biology with Dr. Jerry Hurwitz at our neighboring Sloan Kettering Memorial Research Institute, so our joint department numbers 30 faculty members. We have full access to the core facilities at Sloan Kettering. Dr. Neil Hackett is within our department, and Dr. Mathew Lubin is At the Strang Cancer Cancer Prevention center across the street. In addition, I have an adjunct appointment at The Rockefeller University (also across the street) in the Department of Chemistry, Biochemistry, and Structural Biology, now headed Dr. David Cowburn. Finally, my X-ray collaborator, Dr. Aneel Aggarwal is a few miles uptown at the College of Physicians & Surgeons, Columbia University. Thus, we can all easily get together to discuss new results and ideas.

**RESOURCES AND ENVIRONMENT**

FACILITIES: Mark the facilities to be used at each performance site listed in Item 9, Face Page, and briefly indicate their capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Use "Other" to describe the facilities at any other performance sites listed in Item 9 on the Face Page and at sites for field studies. Use continuation pages if necessary. Include an explanation of any consortium/contractual arrangements with other organizations.

Q Laboratory: Second Floor, Black Building, Columbia Health Sciences Campus:  
 630 square feet laboratory with 6 benches and desk space for six persons.  
 This will be sufficient for for most biochemical work, room temperature crystallization, and crystal mounting.  
Other:  
 Access to cold rooms, dark rooms, and X-ray facilities.

Q Clinical:

Q Animal:

Q Computer: Iris Indigo XZ24 for computing and graphics  
 Access to Convex C220 for general purpose computing.  
 Access to VAX 750 for reading magnetic tapes.  
 Access to GPX Vaxstation II for data processing

Q Office: A 430 square feet of office space: PI's office (120sq. ft.) + computer terminal room (310 sq. ft.) on the second floor of Black Building.

Q Other (Cancer Center ): Access to core facilities of Columbia Cancer Center

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

2 Rigaku X-ray generators	Black Bldg. 2nd Floor
1 Xuong-Hamlin Area Detector	"
2 Huber Precession Cameras	"
1 Applied Biosystems DNA Synthesizer	"
1 Pharmacia FPLC System	"
1 lyophilizer + 1 Servant Speedvac	"
1 Sorvall RC5 Centrifuge	"
1 -70°C Freezer	"

ADDITIONAL INFORMATION: Provide any other information describing the environment for the project. Identify support services such as consultant, secretarial, machine shop, and electronics shop, and the extent to which they will be available to the project.

The X-ray facilities described above are shared with Dr. Wayne Hendrickson's laboratory.

**A. SPECIFIC AIMS:**

(i) **To construct mutant thermostable ligases and test for greater specificity.** A systematic site-specific mutagenesis approach has pinpointed several residues of the *Tth* ligase gene which play a role in adenylation or enzymatic joining of adjacent, perfectly matched oligonucleotides. An assay has been developed to screen mutant ligases for enhanced specificity in discriminating a perfect match from a single-base mismatch at the ligation junction. Determination of the three-dimensional structure of *Tth* ligase, and of *Tth* ligase/DNA complexes will aid significantly in the design of new mutants. A mutant ligase with increased specificity in discriminating matched from mismatched primers will increase the sensitivity of our PCR/LDR detection of cancer mutations (See Projects 1 and 2).

(ii) **To test modified oligonucleotides for greater specificity during ligation.** An assay has been developed to test the specificity of *Tth* ligase in discriminating a perfect match from a single-base mismatch at the ligation junction. Introducing a nucleotide analogue (See Project 3) or mismatched base adjacent to or near the discriminating base may increase the specificity of this reaction. Such modified oligonucleotide primers will be tested in our fidelity assay using wild type and mutant *Tth* ligase. Incorporating modified oligonucleotides in primers to improve the fidelity of *Tth* ligase in discriminating matched from mismatched primers will increase the sensitivity of our PCR/LDR detection of cancer mutations (See Projects 1 and 2).

(iii) **To determine the three-dimensional structure of *Tth* ligase and of *Tth* ligase/DNA complexes.** *Tth* ligase has been cloned and overproduced to greater than 10% of *E. coli* cellular proteins. Large quantities of the enzyme will be purified for crystallization in the presence and absence of its cofactor nicotinamide adenine dinucleotide (NAD). The crystals will be used to determine the 3-D structure of the ligase by X-ray crystallography. The structure will help provide a structural understanding of its thermostability. The enzyme will also be crystallized in association with a nicked DNA duplex. This structure will help to reveal the mechanism of DNA ligation, and provide an understanding of the specificity of the enzyme towards mismatches at the nicked site. Solving the three dimensional structure of *Tth* ligase will aid in designing new mutants with greater specificity.

**B. BACKGROUND AND SIGNIFICANCE**

Since its discovery in 1985, the polymerase chain reaction (PCR) has had a profound impact on detecting genetic and infectious diseases, identifying new genes, and unraveling the mysteries of protein-ligand recognition [1-6]. Its universal utility is due to the exquisite sensitivity of amplification and the ease of cycling made possible by the cloning and careful characterization of a thermostable polymerase from *Thermus aquaticus* [7-10]. Likewise, cloning of thermostable ligase offered a new amplification method, termed ligase chain reaction (LCR), to both amplify DNA and discriminates single base mutations [11-13]. Allele-specific LCR employs four oligonucleotides: two adjacent oligonucleotides which uniquely hybridize to one strand of target DNA, and a complementary set of adjacent oligonucleotides which hybridize to the opposite strand (See Fig. in overview). Thermostable DNA ligase covalently links each set, provided that there is complete complementarity at the junction [12]. Since the oligonucleotide products from one round serve as substrates during the next round, the signal is exponentially amplified, analogous to PCR amplification. A single-base mismatch at the oligonucleotide junction will not be amplified, and is therefore distinguished.

Single base mutations may also be detected using allelic specific PCR, nested PCR, PASA or double ARMS amplification [10, 14-19]. Yet diagnostic use in the clinic has met certain limitations. PCR requires careful optimization to detect a cancer mutation in the presence of normal tissue. Furthermore, PCR is not optimal for simultaneous detection of several mutations in a single reaction (known as multiplexing). When several mutations are on a single gene, such as the p53 tumor suppressor gene, multiple PCR primers may run into each other during amplification. These problems can be overcome by combining LCR with PCR.

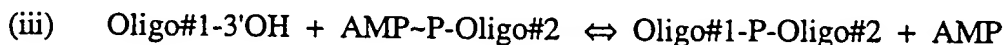
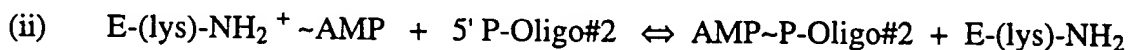
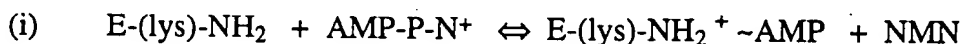
The ligase chain reaction is ideal for multiplexing. Since there is no polymerization step, several primer sets can ligate along the length of a gene without interference. The optimal multiplex detection scheme involves a primary PCR amplification, followed by either an LCR (four primers, both strands) or LDR (two

primers, same strand) detection (See Fig. 3). This approach has been successfully applied to multiplex detection of cystic fibrosis [20-22], hyperkalemic periodic paralysis [23], and 21 hydroxylase deficiency (D. Day, P. White, and F. Barany, unpublished). In these examples, homozygous normal, heterozygous, and homozygous disease individuals are distinguished. Reliable detection of cancer mutations will require finding mutations present in a minority of the cells from tissue biopsies. This project seeks to optimize conditions and/or use mutant enzymes to increase the sensitivity of direct PCR/LDR detection of mutations in the 1 in  $10^2$  to 1 in  $10^3$  cell range.

PCR amplification exploits two primers to obtain three types of information: (i) the presence of target sequence, (ii) the distance between primers, and (iii) the sequence between the primers. LDR exploits two primers to obtain only two types of information: (i) the presence of adjacent target sequences, and (ii) the presence of perfect complementary to the primers at the junction of these sequences. LCR exploits four primers to obtain the same information as LDR, while also achieving signal amplification. LDR, LCR, and PCR amplification derive their specificity in part from the initial hybridization of primer to target DNA. This specificity is enhanced by: (i) using oligonucleotides of sufficient length so they are unique in the human genome, and (ii) using temperatures near the oligonucleotide  $T_m$ . With PCR, background target-independent amplification results in primer-dimers, which are of lower molecular weight than the desired product and thus easily distinguished. However, with LCR, background target-independent amplification products are the same size as the desired products. Hence, to make LCR practical, it was necessary to completely eliminate target independent ligations. This was accomplished only with use of thermostable ligase [12]. LDR only provides linear amplification, but it does not yield target independent ligation products. Combining PCR with LDR joins the best advantages of both techniques. PCR provides sensitivity in being able to amplify as few as one starting target sequence. LDR detects the presence of this amplified sequence, without giving target independent signal. Furthermore, LDR allows for the simultaneous detection of dozens of closely spaced mutations without interference between primers. Thus, by increasing the specificity of the LDR reaction we aim to simultaneously screen for dozens of potential mutations which may be present at less than 1% of the tumor biopsy.

There are three basic approaches to increasing the specificity of an enzymatic reaction: (i) modify the reaction conditions. (ii) modify the substrate, and (iii) modify the enzyme. The latter approach will be greatly aided by determination of the three dimensional structure of *Tth* ligase. This project combines all three approaches.

The mechanism of DNA ligase is composed of three discrete and *reversible* steps: (i) formation of a high-energy enzyme intermediate by transfer of the adenosyl group from NAD (or ATP) to the  $\epsilon$ -amino group of a lysine residue; (ii) transfer of the adenosyl group to the 5' phosphate of one DNA strand forming an activated pyrophosphate linkage; and finally (iii) attack of this activated 5' end by a 3'-hydroxyl group on the adjacent DNA strand, thus forming a phosphodiester link between the two DNA strands, and eliminating AMP [24-32]. These steps are shown below for the *E. coli* or *T. thermophilus* DNA ligase, and depicted in Fig.1.



Initial experiments in the P.I.'s laboratory demonstrated the exquisite fidelity of *Tth* ligase. The enzyme demonstrated a 50 fold discrimination between an A:T match and a G:T mismatch, and greater than 500 fold discrimination between an A:T match and an A:A mismatch [12]. We have recently developed a rapid and more sensitive assay for systematically measuring the fidelity of wild-type and mutant *Tth* ligases. Results show that wild type enzyme exhibits superior discrimination between two alleles if the discriminating base is on the 3' end as opposed to the 5' end of the oligonucleotide (see Fig. 4 & 5, Preliminary results). Even greater specificity may be achieved by introducing a nucleotide analogue or mismatched base near the ligation junction (see Fig. 1B, and Experimental Design and Methods section ii.).

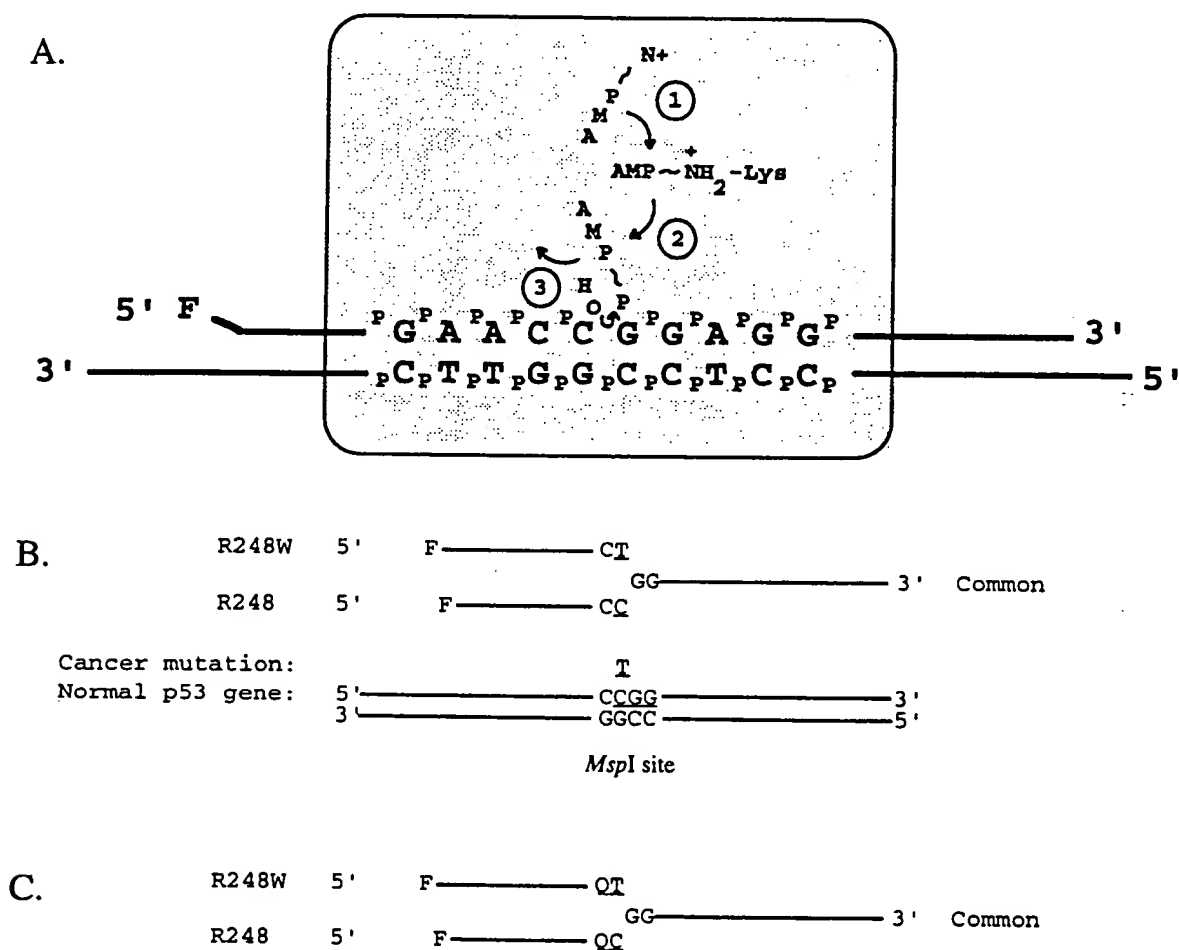


Fig. 1. Detection of a site-specific mutation in codon 248 of p53 gene with Tth ligase-directed LDR. **A:** The catalytic mechanism of nick-closure in a DNA duplex by Tth ligase. Step 1: Tth ligase interacts with co-factor,  $\text{NAD}^+$ , to form a covalent enzyme-adenylate intermediate. Step 2: The AMP moiety is transferred to the 5'-terminal phosphate of the DNA strand break. Step 3: The nick (a single-stranded break in DNA duplex) is closed by the formation of a 3'-5'-phosphodiester bond after the attack of the 5'-adenylated phosphate by the 3'-hydroxyl group which releases AMP. **B:** Detection of a site-specific mutation in codon 248 of p53 gene with fluorescent LDR. A transition mutation, C to T, in codon 248 of p53 (which is part of an MspI site) has been found in many cancers, causing a change from Arg to Trp. In order to detect this mutation, three oligonucleotides are designed for the fluorescent LDR test. The two up stream oligonucleotides (R248W and R248), both labeled with a fluorescent dye at the 5'-end, are designed to detect the mutant and wildtype target respectively. Their lengths are varied so that they will form different sized-product after ligating to the common down stream oligonucleotide. **C:** A nucleotide analog (Q) is placed adjacent and upstream of the detecting base in the fluorescent detecting oligonucleotides for possible improvement of ligation fidelity in LDR tests.

Protein engineering of the *Tth* ligase will be greatly aided by determination 3-dimensional structure of the enzyme-DNA substrate using X-ray crystallography. This method has proved to be a powerful technique in the study of protein-DNA interactions. Since 1984, well over a dozen protein-DNA complexes have been crystallized and their structures determined at atomic resolution. The proteins range from prokaryotic and eukaryotic transcription factors to bacterial restriction enzymes [33-36]. Many of the proteins contain repeating motifs or domains such as helix-turn-helix, zinc-finger regions and others. A comparison of structures reveals common features of DNA binding proteins.

In most cases, a secondary structural element such as an  $\alpha$ -helix or  $\beta$ -sheet is inserted into the major groove of the DNA and specific hydrogen bonds are formed between the side chains and the polar groups on the base pairs. Non-polar contacts, especially to the 5-methyl groups of thymine bases, also appear to be

important in defining specificity. Some interactions, such as between amino acid arginine and base guanine and amino acid glutamine and base adenine, were predicted earlier [37], whilst others such as lysine to guanine, glutamine to thymine, or glutamate to cytosine for instance were unanticipated [33, 38].

In addition to these "specific" interactions, all of the proteins make extensive contacts with the sugar-phosphate backbone of the DNA [33, 34, 38]. These contacts may be the first to form on DNA binding, and may help to establish the correct register between the protein and the DNA. The contacts can often be highly conserved within a family of proteins. In lambda and 434 phage repressors, a glutamine at the start of helix 2 and an asparagine at the end of helix 3 make similar contacts to the DNA backbone, helping to anchor the recognition helices with similar orientations and positions in the two protein-DNA complexes [35]. For proteins that interact non-specifically with DNA, contacts to the DNA backbone may be the primary mode of DNA recognition. Amino acids that are frequently found to interact with the sugar-phosphate backbone in protein-DNA structures are arginine, lysine, glutamine, asparagine, threonine, tyrosine, and histidine. All of these residues possess potential hydrogen bond donor groups, which often interact with the non-esterified phosphate oxygens on the DNA. Positively charged arginine and lysine residues can also help to stabilize the negative charge of the sugar-phosphate backbone, and are the most common residues found in association with the DNA backbone.

To consider a few protein-DNA structures: In complexes of EcoRV with cognate and non-cognate DNA sites, majority of the contacts to the DNA backbone appear to be formed by lysine and arginine residues. Lys38, Lys102, Lys109, and Arg140 make contacts with the backbone in both complexes, while the active site residue Lys92 makes contacts only in the complex with the cognate DNA site [39]. In the structure of the GCN4-DNA complex, 13 of the 17 amino acids of the protein dimer that contact the DNA backbone are lysines or arginines [40]. Similarly, in the structure of GAL4 with DNA over 80% of the interactions with DNA backbone are formed by lysines and arginines [41]. In the structure of glucocorticoid receptor with DNA [42], at least of the half of the amino acids forming hydrogen bonds with the DNA backbone are lysines and arginines. As a final example, 5 of the 9 hydrogen bonds formed with the DNA backbone by the three zinc-finger fragment from Zif268 are with arginine residues [43]. Despite the frequency of these interactions, it should be emphasized that not all the lysine and arginines in a protein are involved in DNA contacts. In the absence of a three dimensional structure there are also no rules for predicting which lysines or arginines are likely to be involved in DNA contacts.

## C. PRELIMINARY RESULTS

### (i) Ligase chain reaction and ligase detection studies:

(a) *Genetic disease detection and DNA amplification using cloned thermostable ligase.* Thermostable DNA ligase was cloned and harnessed in an assay that both amplifies DNA and discriminates single base substitutions. This cloned enzyme specifically links two adjacent oligonucleotides when annealed at 65°C to a complementary target, only if the nucleotides are perfectly base paired at the junction. Oligonucleotide products are exponentially amplified by thermal cycling of the ligation reaction in the presence of a second set of adjacent oligonucleotides, complementary to the first set and the target. A single base mismatch prevents ligation/amplification and is thus distinguished. This method was exploited to detect 200 target molecules as well as to discriminate between normal  $\beta^A$  and sickle  $\beta^S$  globin genotypes from 10 $\mu$ l blood samples.

The P.I.'s laboratory, in collaboration with others, has used PCR coupled with LDR/LCR detection to easily discriminate single base mutations in Leber's Hereditary Optic Neuropathy, *Listeria monocytogenes*, hematopoietic tumors, and Hyperkalemic Periodic Paralysis [12, 13, 23, 44-48]. More recently, we have extended PCR/LDR to detect congenital adrenal hyperplasia which results from 21-Hydroxylase deficiency. We have developed methodologies which rapidly determine heterozygous or homozygous individuals for any of the ten common gene conversions in *CYP21*. Allele specific PCR was used to amplify defined regions of *CYP21*. A subsequent ligase detection reaction (LDR) determined whether the *CYP21* (active wild-type) or *CYP21P* (pseudo gene) sequence was present in a particular *CYP21*-specific PCR amplification. The LDR primers were designed such that the upstream oligonucleotide is fluorescently labeled and complementary at its 3' end to either *CYP21* or *CYP21P*. Ligation to a common unlabeled primer (the downstream LDR

oligonucleotide) only occurred if there was a perfect match at the 3' end of the upstream oligonucleotide. By using different length fluorescent primers for the pseudo gene and active gene, different length LDR products were formed. Twenty alleles were differentiated and quantified using an ABI 373A DNA sequencer with Genescan 672 software.

## (ii) Thermostable ligase protein studies:

(a) *Cloning, overexpression and nucleotide sequence of a thermostable DNA ligase-encoding gene.* The *Thermus thermophilus* (*Tth*) DNA ligase-encoding gene (*ligT*) was cloned in *Escherichia coli* by genetic complementation of a *ligts7* defect in an *E. coli* host. Nucleotide sequence analysis of the gene revealed a single chain of 676 amino acid residues with 47% identity to the *E. coli* ligase. Under *phoA* promoter control, *Tth* ligase was overproduced to greater than 10% of *E. coli* cellular proteins. Adenylated and deadenylated forms of the purified enzyme were distinguished by apparent molecular weights of 81 kDa and 78 kDa, respectively, after separation by sodium dodecyl sulfate-polyacrylamide-gel electrophoresis. The nucleotide sequence of the *Tth* ligase gene was determined in collaboration with Dr. David Gelfand of Roche Molecular Systems, who continues to interact with us on our ligase studies (see letter of collaboration in overview section of program project.)

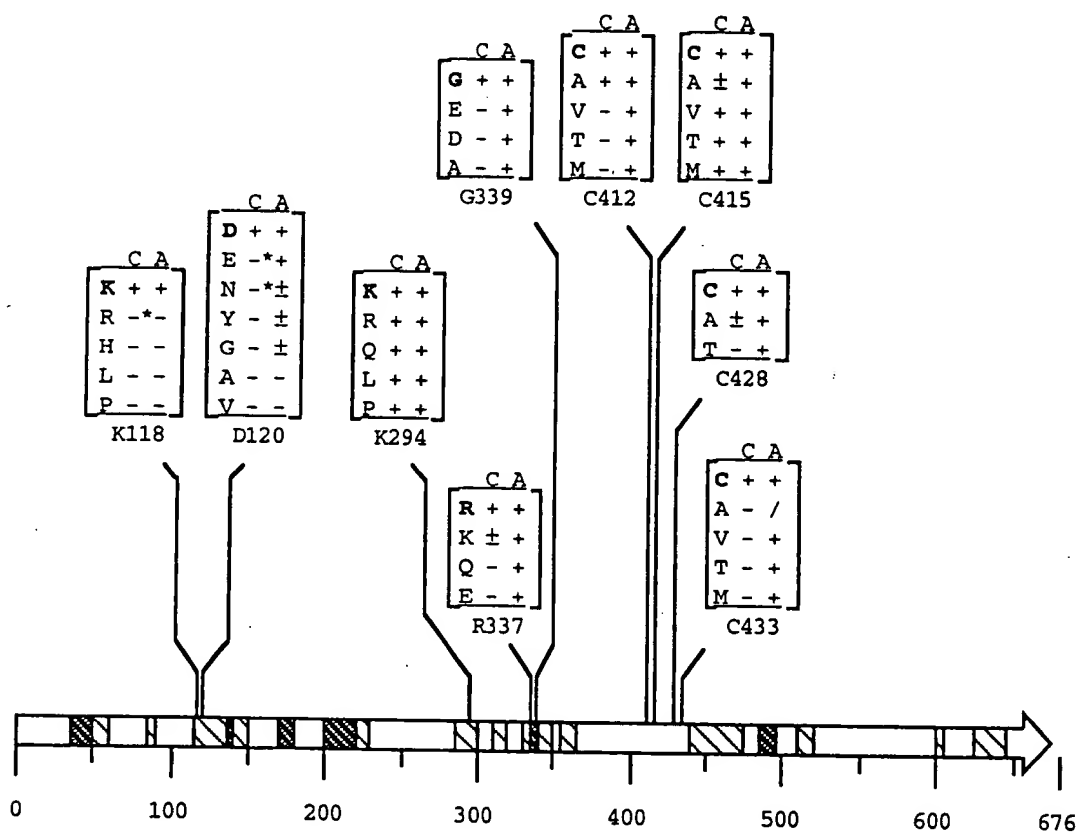


Fig. 2. Effects of site-specific mutagenesis on *Tth* ligase activity: The horizontal white arrow represents the full-length *Tth* ligase protein. Homologous regions between *T. thermophilus* ligase, *E. coli* ligase, and *Z. mobilis* ligase are indicated as hatched areas on the white arrow. Darker hatched areas indicate stronger homology. Site-specific mutagenesis of amino acid residues are shown by vertical lines drawn from the arrow. The effects of mutations at a given site are summarized in a mini-table. C = *in vivo* complementation of *ts lig* host; A = *in vitro* adenylation; "+" = similar activity to wildtype enzyme; "-" = no activity; "±" = partial activity or intermediate activity; "/" = not tested, enzyme lost thermostability. Complementation of a temperature sensitive *E. coli* host was assayed by introducing plasmids containing a mutant *Tth* ligase gene into strain AK76 (*lig ts7*), and replica-plating transformants at 32°C and 42°C. For adenylation assays, purified mutant ligase proteins were incubated in the presence of NAD<sup>+</sup> at 64°C for 25 min. Adenylated and deadenylated forms of the mutant enzyme were well separated on a 7.5% SDS polyacrylamide gel. \*: Compared to the wildtype enzyme, mutant D120E has a very high activity in adenylation, but a very weak activity in deadenylation. In addition, although mutants K118R, D120E, and D120N lacked *in vivo* complementing activity, they all showed low levels of *in vitro* DNA nick-closing activity.



(b) *Identification of the adenylation site of Tth DNA ligase by site-directed mutagenesis.* To identify active site residues, a series of site-directed *Tth* ligase mutants were constructed using an overlap extension PCR method [49]. A protein mini-prep method was developed to prepare mutant *Tth* ligase for an *in vitro* adenylation assay. Induced cells (from a 6 ml culture) were sonicated and the cell lysate was heat treated to denature and precipitate *E. coli* proteins. The supernatant contained approximately 200µg of 70% pure *Tth* ligase for biochemical characterization (J. Luo and F. Barany, unpublished results). Results with *in vivo* complementation of an *E. coli* ts lig host and an *in vitro* adenylation assay are summarized in Fig. 2 above. K118 was found to be the active site lysine for the adenylation of the *Tth* ligase, while K294 was shown to be non-essential for the ligase activity. Mutations at D120 indicate that this residue is not only involved in adenylation, but also in the deadenylation and ligation steps. These two residues are part of a conserved motif, KVDG, which others have suggested as important in adenylation [50]. In addition, R337, G339, C412, and C433 were also found to be important for the activity of the *Tth* ligase, but have no effect on the adenylation or deadenylation steps. The cysteine residues may play a role in DNA binding, as recently described for a zinc ribbon [51]. Biochemical characterization of wildtype and mutant *Tth* ligase discrimination of perfect match from single base mismatches will be determined as described below.

### (iii) Assay of ligase fidelity.

(a) *Development of a rapid assay to test fidelity of wild type and mutant ligases.* Initial experiments in a carefully defined assay system used a 61 nucleotide oligonucleotide as the bottom (template) strand. Two different size discriminating oligonucleotides were labeled at their 5' ends with <sup>32</sup>P. A common upstream oligonucleotide forms perfect Watson-Crick base-pairing to the template, while one of the detecting oligonucleotides has a single mismatch at the 5' nucleotide (G:T) and the other oligonucleotide has a perfect match (A:T). Ligations were for five cycles of one minute at 94°C, followed by four minutes at 68°C in a Perkin Elmer 9600 machine. Products were analyzed by electrophoresis on 10% acrylamide-urea gels and quantified using a Molecular Dynamics phosphorimager. The perfect match oligonucleotide was converted to 50 to 100% product, and was clearly visible. However, the mismatch ligation product was below the phosphorimager resolution, such that the upper limit for the ratio of matched to mismatch ligations could not be measured. Thus accurate quantification with radioactive detection would necessitate time consuming excision of product bands from the gel, as was done in our initial LDR assays [12].

We therefore developed a fluorescent assay to determine ligation rates at an isothermal temperature. Data generated from these studies will be applied to optimize thermal cycling conditions used in LDR. The templates and detecting oligonucleotides for this assay were designed so their T<sub>m</sub>'s were sufficiently higher than the assay temperature of 65°C. This would avoid problems associated with oligonucleotides melting and re-annealing. As with the previous assay we generated a nicked duplex substrate by annealing two adjacent oligonucleotides to a longer complementary template (bottom) strand. Bases at the junction are either perfectly base-paired or have a single mismatch on either the 3' or 5' side of the nick. We have made a matrix of 14 oligonucleotides to generate all the possible combinations of different base pair matches and mismatches at the 3' and 5' sides of the nick (see Fig. 3). By using a saturating amount of substrate (nicked duplex DNA formed by the three oligonucleotides) and sufficient enzyme to give linear rates, we are trying to determine the specific activity of *Tth* ligase (wild-type and mutants) with our matrix of nicked duplex substrates. Since the experiment is isothermal, we can extend the ligation times to many hours allowing for accurate quantification of mismatch ligation products. Although radioactive detection is capable of giving very high sensitivities, we are using fluorescently labeled primers. These offer the advantage of stability and incorporation of equimolar amounts of fluorescent dye during synthesis. An Applied Biosystems 373A DNA sequencer with Genescan 672 software is used to detect and quantify products (See Core B). Currently using this system we are able to detect as little as 100 attomole of product, with a linear detection range covering two orders of magnitude. The slight decrease in sensitivity compared with <sup>32</sup>P labeling can be readily overcome by using longer reaction times or using more enzyme within the linear range of the assay.

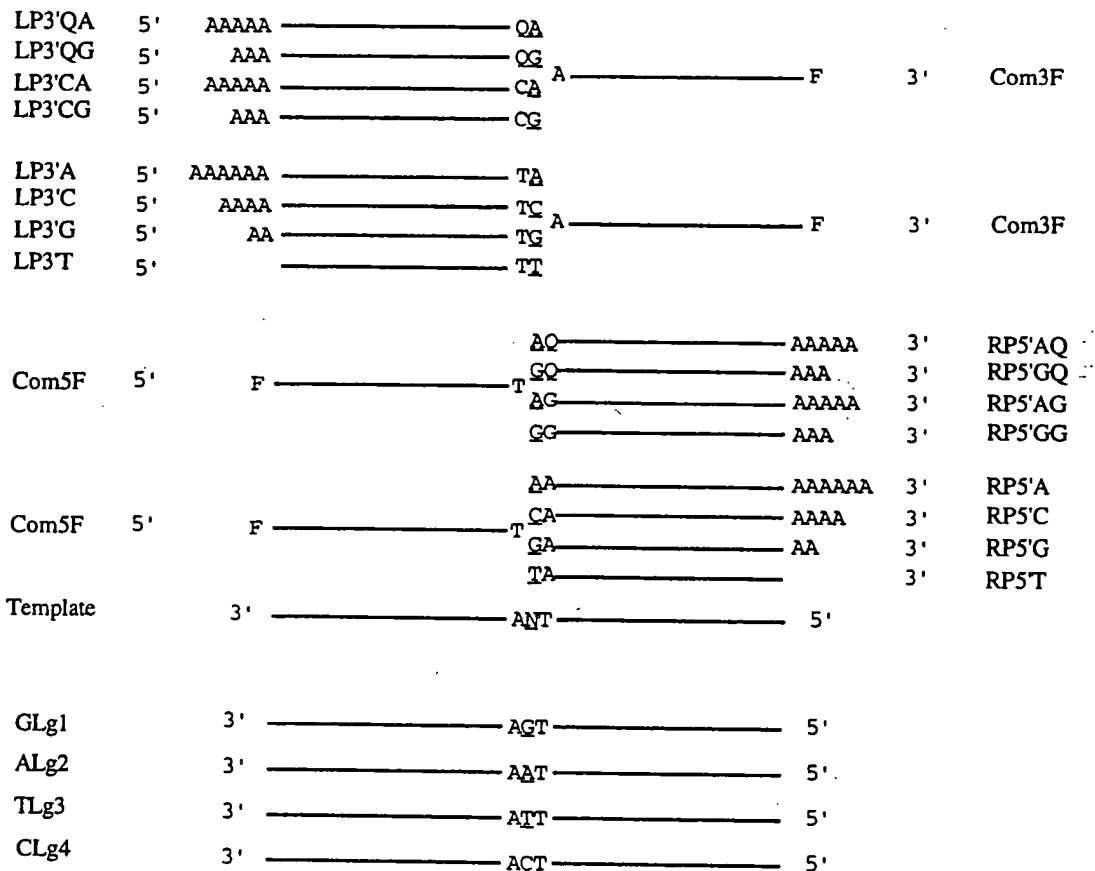


Fig. 3. LDR primers for testing the fidelity of wild type and mutant *Tth* ligase. Schematic diagram of synthetic primers and templates for testing ligase fidelity. A set of 14 oligonucleotides can test for ligation of any possible base pair match or mismatch. Oligonucleotides LP3'A, C, G, and T contain the discriminating nucleotide on the 3' strand and may be ligated to fluorescently labeled Com3F in the presence of perfectly complementary, or mismatched template G, A, T, and CLg4. Likewise, oligonucleotides RP5'A, C, G, and T contain the discriminating nucleotide on the 5' strand and may be ligated to fluorescently labeled Com5F in the presence of template. The discriminating oligonucleotides have different length poly A tails to separate products on an ABI 373A DNA sequencer. The primers are designed with  $T_m$  values well above the assay temperature of 65°C. Primers LP3'CA, LP3'CG, RP5'AG, and RP5'GG each contain an intentional mismatch at the base adjacent to the discriminating base. Similar primers LP3'QA, LP3'QG, RP5'AQ, and RP5'GQ have been designed to contain a  $Q_2$  nucleotide analogue adjacent to the discriminating base (See Project 3 for the structure and synthesis of the  $Q_2$  nucleotide analogue). This might increase the selectivity of *Tth* ligase in discriminating single base mutations at the oligonucleotide junction. Sequences of oligonucleotides are available upon request.

Preliminary data using this fluorescent assay indicate that *Tth* ligase covalently closes perfectly matched substrates at a significantly higher rate than mismatched substrates. In addition, the enzyme demonstrates greater specificity for perfectly matched substrate over mismatched substrate when the discriminating base is on the 3' side versus the 5' side of the LDR primer (See Fig. 4 and 5).

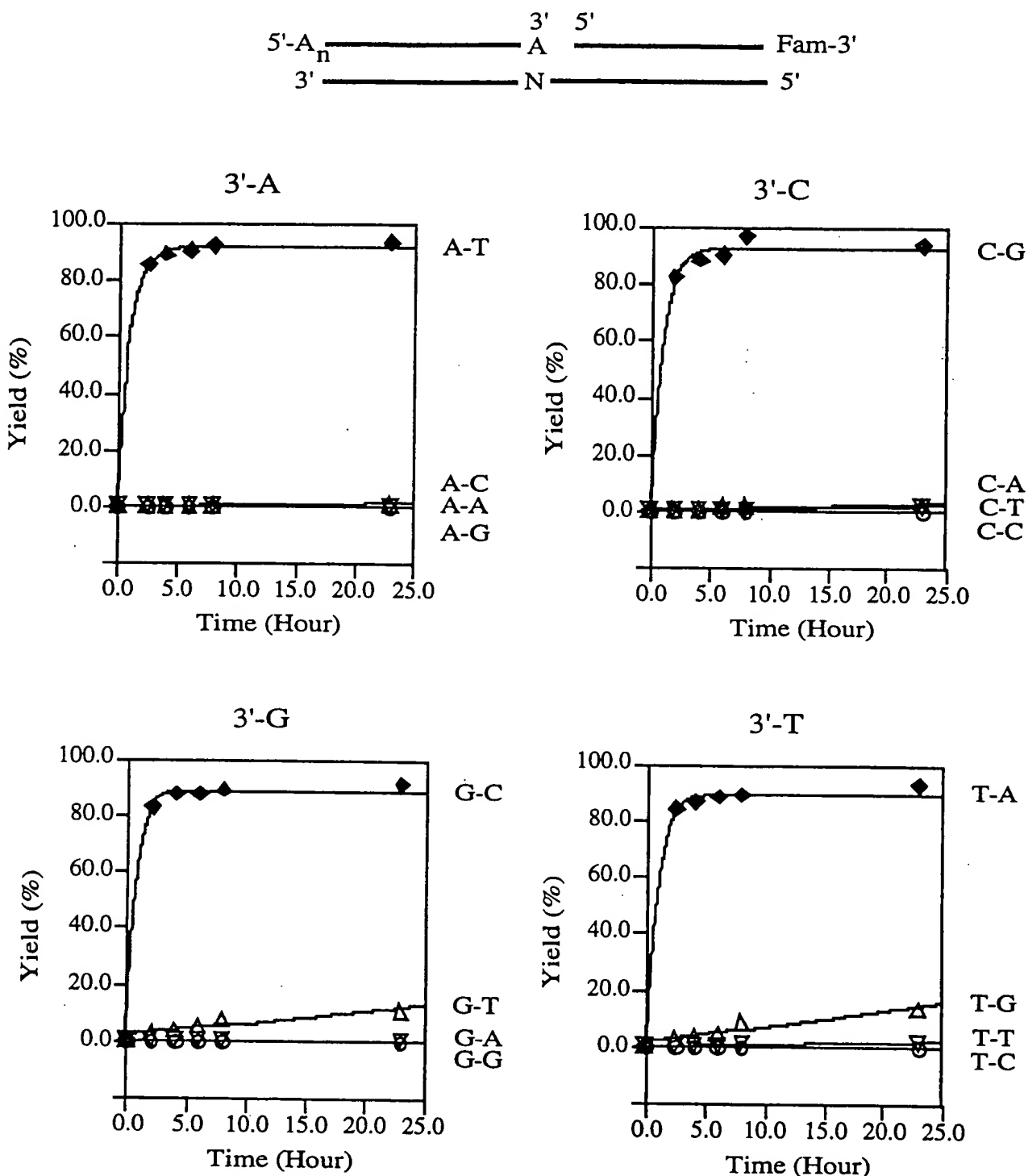
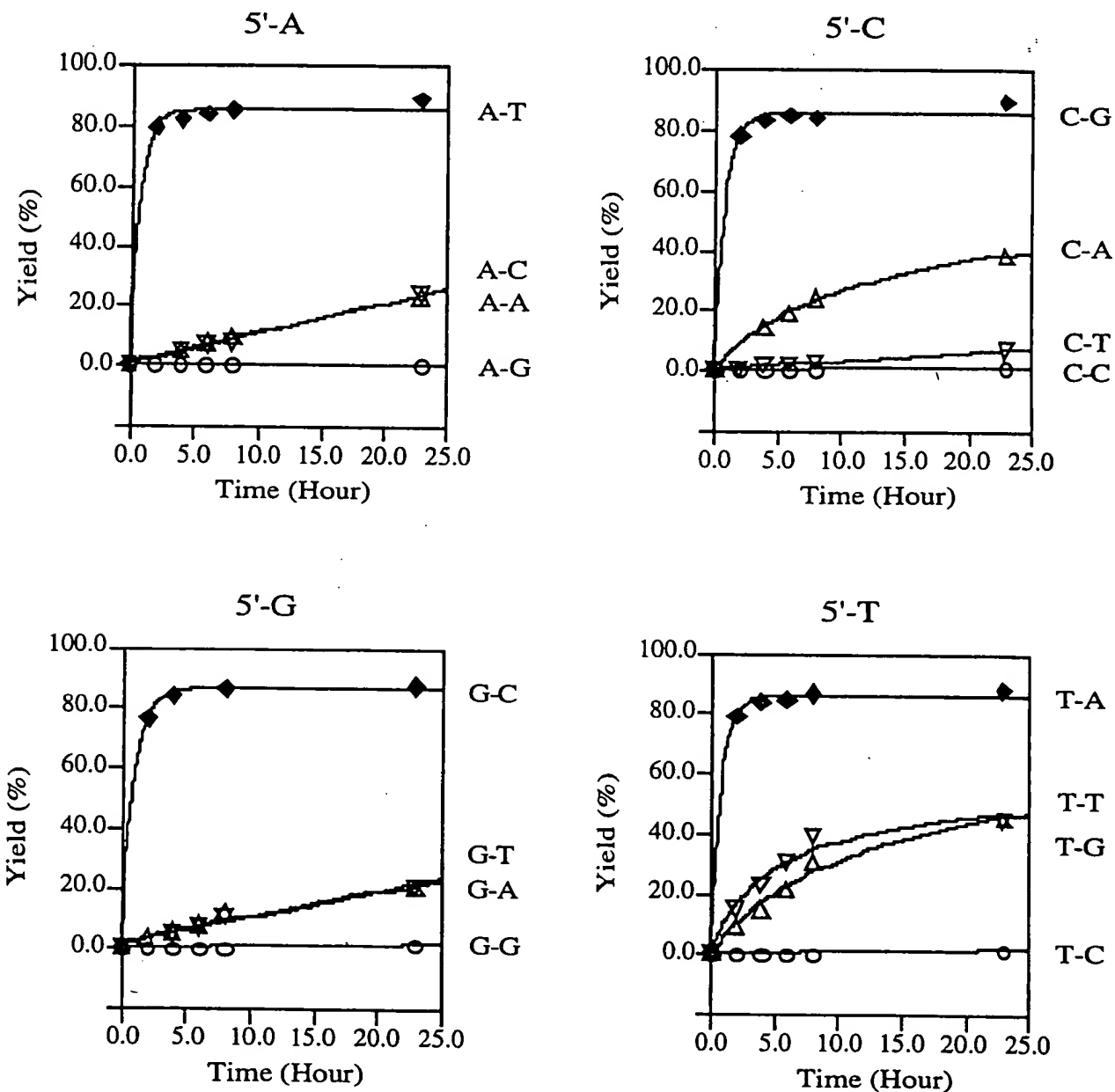
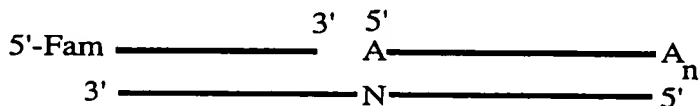


Fig. 4. Fidelity of nick closure by a thermostable ligase at the 3' side of the nick. The ligase substrate (nicked DNA duplex), shown at the top of the figure, is formed by annealing two adjacent oligonucleotides (about 30-35 nt long) to a longer complementary oligonucleotide (59 nt). For clarity, the upstream oligonucleotide at the 3' side of the nick is the detecting oligonucleotide while the downstream FAM-labeled oligonucleotide on the 5' side of the nick is the common oligonucleotide. The lower oligonucleotide is the template strand. The FAM-labeled common oligonucleotide was made by using a 3'-Amino-modifier C3-CPG column from Glen research for the initial DNA synthesis, and the FAM group was then attached through the 3'-amino group using the activated NHS-FAM from the Applied Biosystem Inc. All oligonucleotides were gel-purified and the common oligonucleotide was phosphorylated. The discriminating base "A" in a detecting oligonucleotide and the "N" in a template were varied to give all 16 possible combinations of base-pairing. Each reaction was performed in 40  $\mu$ l of buffer containing 20 mM Tris-HCl, pH 7.6; 10 mM  $MgCl_2$ ; 100 mM KCl; 10 mM DTT; 1 mM  $NAD^+$ ; and 250 fmol of nicked duplex

substrates. Ligations were performed at 65°C, and were started by the addition of 2.5 fmol of the thermostable ligase. 5 µl aliquots were removed at 0hr, 2hr, 4hr, 6hr, 8hr, and 23 hr, and mixed with 18 µl of a stop solution (83% Formamide, 8.3 mM EDTA, and 0.17% Blue Dextran). 5 µl of this mixture was denatured at 93°C for 2 min, chilled rapidly on ice prior to loading on an 8 M urea-10% polyacrylamide gel, and electrophoresed on an Applied Biosystem Inc.'s 373A DNA Sequencer. Fluorescently labeled ligation products were analysed and quantitated using ABI's Genescan 672 software, and the results were plotted using DeltaGraph Pro3 software.



**Fig. 5. Fidelity of nick closure by a thermostable DNA ligase at the 5' side of the nick.** Reaction conditions were basically the same as in Figure 1 except that different detecting and common oligonucleotides were used. The detecting oligonucleotide was on the 5' side of the nick, and phosphorylated. The common oligonucleotide was on the 3' side of the nick, and was 5'-labeled with FAM using 6-FAM amidites from the Applied Biosystem Inc. Analysis of ligation products was as described in Figure 4.

Of the 12 possible mismatches at either side of the nick, some mismatches are discriminated more efficiently than others. For example, a C-C mismatch at the 5' side of the nick, produced less than a 1% yield after 23 hours of incubation, while a C-T mismatch gave a 6.5% yield and a C-A mismatch a 38% yield (See Fig. 5). At present we do not have sufficient data points for the mismatch ligations to know if the same final yield of ligation product would be obtained as with the perfect match, if the ligation was left for sufficiently longer time. Our present data suggests that the same final yield would *not* be obtained, indicating the reverse reaction of ligase catalyzed hydrolysis of mismatched ligations (i.e. nicking) is occurring. The final yield of product would depend upon the relative rates for these two competing reactions. Experiments are underway to investigate a potential proofreading nicking activity of *Tth* ligase.

#### (iv) Studies on the structure of DNA recognition proteins.

Much of the research in the Co-I's laboratory is focused on the structure determination of restriction endonucleases by X-ray crystallography. We have recently completed the 3-D structure of restriction enzyme *Bam*HI, at a resolution of 1.95Å [52]. The structure was determined by application of the new and powerful method of Multiwavelength Anomalous Diffraction (MAD) [53]. The five methionine residues of the enzyme were replaced by selenomethionines, and X-ray data were collected at wavelengths near the absorption edge of Se and at a remote point. Phase information was derived from this and other data by a combination of MIR and MADSYS approaches.

The central core of *Bam*HI turns out to be strikingly similar to the core of *Eco*RI [54]. This was unexpected due to the lack of any amino acid sequence similarity between the two enzymes. Many of the active-site and DNA recognition features appear to be conserved among the two enzymes. It appears that the two enzymes may have diverged from a common ancestor.

### D. EXPERIMENTAL DESIGN AND METHODS

(i) **To construct mutant thermostable ligases and test for greater specificity.** A systematic site-specific mutagenesis approach has pinpointed several residues of the *Tth* ligase gene which play a role in adenylation or enzymatic joining of adjacent, perfectly matched oligonucleotides. An assay has been developed to screen mutant ligases for enhanced specificity in discriminating a perfect match from a single-base mismatch at the ligation junction. Determination of the three-dimensional structure of *Tth* ligase, and of *Tth* ligase/DNA complexes will aid significantly in the design of new mutants. A mutant ligase with increased specificity in discriminating matched from mismatched primers will increase the sensitivity of our PCR/LDR detection of cancer mutations (See Projects 1 and 2).

(a) *Optimization of the rapid assay to test the fidelity of wild type and mutant ligases.* Our preliminary data already suggests that some mismatches are discriminated more efficiently than others. The enzyme demonstrated a 50 fold discrimination between an A:T match and a G:T mismatch, and greater than 500 fold between an A:T match and an A:A mismatch [12]. Our fluorescent assay system will allow us to address questions such as how salt concentration, pH and the presence of detergents alter the fidelity of ligation. Initial studies using *Tth* ligase in LDR experiments has shown how the yield of product varies with buffer conditions (D. Day and F. Barany, unpublished results.) The fluorescent assay will allow us to determine how ligase fidelity varies with the reaction conditions. For the detection of mutations and cancers we are currently limited by our ability to discriminate signal derived from a few cells carrying the cancer DNA mutation from mismatch ligation signal generated by low efficiency ligation on normal DNA template.

Initial studies have already shown that the enzyme demonstrates a tighter discrimination of a mismatch when the discriminating base is on the 3' end of the primer. The assay will allow us to test the following variations in experimental design: (i) Should the detecting oligonucleotides compete with each other for binding to the detection site? (ii) Should the ligation temperature be equal to or above the oligonucleotide  $T_m$  in order to promote rapid exchange of the hybridized oligonucleotide to prevent mismatch ligation? We also do not know if there is a difference when the nicked duplex substrate is formed by two detecting oligonucleotides competing for one target, or when two differing templates compete for one detecting oligonucleotide (such as in cancer detection experiments). We have also shown in LCR experiments that ligation efficiencies of

perfectly base paired nicks are dependent upon the 5' and 3' base at the nick site [12]. The extent of these differences will be determined in the course of this study. These slight differences are important when quantification of target is required, such as when determining whether an individual is heterozygous or homozygous for a given allele. A bias toward ligation of one allele over another in a heterozygous individual could lead to a misrepresentation of the genotype. We aim to answer these sorts of questions and provide the first detailed characterization of factors pertaining to fidelity of LDR.

Determining this basic biochemical data is required in order to know the limits of this powerful technique, as well as to aid in the choice of optimal thermal cycling conditions. This assay is also of key importance for understanding the catalytic mechanism of the ligase, as well as for our site directed mutagenesis studies. We need to thoroughly characterize the mutant ligases we are engineering with respect to altered fidelities and specificities for use in LDR.

Our future experiments will include the following:

(i) Find the optimal pH, salt concentration, incubation time, and temperature that give maximum fidelity for *Tth* ligase in LDR assays.

(ii) Complete our studies in evaluating the ratio of the specific activities for each possible match and mismatch combination.

(iii) Characterize the mutant ligase for altered fidelity on mismatched substrates which are poorly discriminated by wildtype *Tth* ligase. We will repeat the same experiments with our ligase mutants as were performed with the wild type enzyme. It is possible that some of the mutant ligases have increased specificity for a certain type of mismatch but a decreased specificity for others. Also, some of the ligase mutants may have low activity due to perturbation of the adenylation step, although they may have a very high specificity. The system we are using is able to distinguish these subtle differences in enzyme fidelity.

(iv) Compare the effect of two detecting oligonucleotides competing for one target template and the effect of two different templates competing for one detecting oligonucleotide. The latter case is analogous to detecting a minority of cells in a tumor containing a specific cancer mutation. Preliminary results indicate that wild type ligase can discriminate the presence of a perfectly matched template (cancer mutation) that is just 1% of the mismatched template (normal cells).

(v) Study the possible mismatch nicking effect by *Tth* ligase during prolonged incubation or during thermal cycling in LCR and LDR. The amount of the single stranded break made by ligase on double stranded duplex with or without mismatch and the position of the break (on the upper or lower strand, on the 3' or 5' side of the mismatch in the heteroduplex, or adjacent to the mismatch) is important for understanding the mechanism of ligase. In addition this information will be needed to improve specific ligation yields when detecting a minority cancer mutation in the presence of a 1,000 fold excess of wild type DNA. We are aware that site specific nicking of a mismatched heteroduplex by the reverse reaction of *Tth* ligase could be used as a rapid method for identifying new single base mutations in genes.

(b) *Characterizing Tth ligase mutants, and isolating additional mutants with higher fidelities.* The rationale for generating mutants in *Tth* ligase is that DNA recognition enzymes are often over determined. For example, a mutant of *TaqI* restriction endonuclease constructed in the P.I.'s laboratory demonstrated slightly reduced activity for the canonical sequence, but no activity for closely related sequences under "star" conditions [55, 56]. The specificity of the mutant enzyme was thus improved over the wild-type enzyme. One could hypothesize similar increased specificity mutants with *Tth* ligase.

In preliminary results, the P.I.'s laboratory characterized 34 individual mutations in nine different amino acid residues in *Tth* ligase, which are conserved among three bacterial species (See preliminary results, section ii). This approach is based on the principle that identical amino acid residues in conserved regions may play a role in catalysis or binding. Our approach has been validated by identifying active site K118 and D120 residues, which play an important role in adenylation and deadenylation. Preliminary experiments with *in vitro* activity of our *Tth* ligase mutants suggest that K294R, K294P, C415A, and C428A may be candidates for increased specificity in discriminating matched from mismatched targets. We do not yet know where is the rate determining step in the reaction pathway for ligation of a mismatch or a perfect match. For example, it is possible that deadenylation mutants may show increased specificity because they fail to transfer the AMP group to the 5' oligonucleotide phosphate in the presence of a mismatch. We plan to assay these mutants for the fidelity of ligating an A:T match compared to a G:T mismatch (at both 3' and 5' end), using the fluorescent

oligonucleotide substrates described above. This particular mismatch is the most difficult to discriminate, and thus will become our "gold standard" assay of all our ligase mutants. Promising candidate mutant ligases will be further characterized using the entire matrix. The ultimate assay will use dilutions of PCR amplified p53 mutant DNA into wild type DNA. LDR linear amplification using mutant specific oligonucleotides will be performed to determine the limit of detection of a mutant signal above the mismatch "noise" signal generated by the vast excess of normal DNA. We aim to find conditions in which wild-type or mutant *Tth* ligase can discriminate one cancer mutation in  $10^2$  to  $10^3$  normal cells.

Arginine and lysine residues of DNA binding protein often interact with the DNA phosphate backbone (see significance). Therefore additional mutations will be constructed at such conserved residues using overlap PCR mutagenesis as described in the preliminary results (ii). Fig. 6 shows 10 candidate arginine residues and 6 candidate lysine residues which are identical, and in conserved regions in *E. coli*, *T. thermophilus*, and *Z. mobilis* [11, 57]. Of particular interest is the cluster of five conserved lysine residues at the carboxy terminus of the protein. The site-directed mutagenesis work will be tremendously aided by determination of the three dimensional structure of *Tth* ligase and *Tth* ligase/DNA complexes (see below).

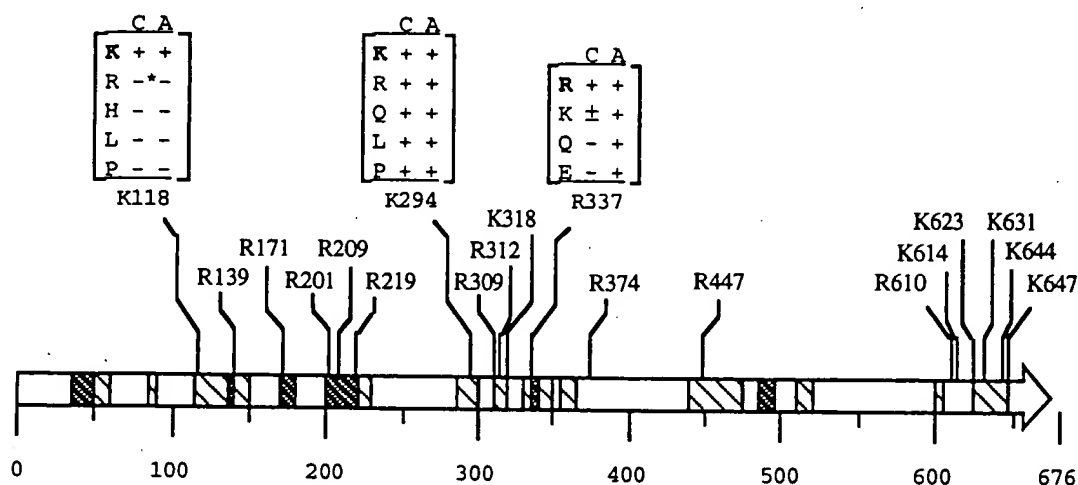


Fig. 6. Proposed areas for further site directed mutagenesis studies. The horizontal arrowed-bar represents the full-length *Tth* ligase protein. Homologous regions between *T. thermophilus* ligase, *E. coli* ligase, and *Z. mobilis* ligase are shown as hatched areas on the arrowed-bar. Darker hatched areas indicate higher homology. K118, K294, and R337 were mutated during the identification of the adenylation site of the *Tth* ligase and the effects of these mutations on enzyme activity at each site are summarized in the mini-tables. Complementation of *ts lig*-host and *in vitro* adenylation are denoted by C and A respectively: similar activity to wildtype enzyme "+"; partial activity or intermediate activity "±"; no activity "-"; not-tested "/". K118 was found to be the adenylation site of *Tth* ligase. Some of the positively-charged amino-acids (Lys and Arg residues) conserved among *T. thermophilus* ligase, *E. coli* ligase, and *Z. mobilis* ligases have been selected for further site directed mutagenesis. Their positions within the ligase protein are indicated by the vertical lines. These residues may play a role in binding the sugar phosphate backbone, and mutations in these positions may alter *Tth* ligase specificity.

If this work leads to the isolation and characterization of a mutant *Tth* ligase with improved discrimination of mismatched bases over wild type enzyme, the mutant enzyme would be incorporated into our multiplex PCR/LDR schemes for even more sensitive detection of cancer causing mutations.

(ii) **To test modified oligonucleotides for greater specificity during ligation.** An assay has been developed to test the specificity of *Tth* ligase in discriminating a perfect match from a single-base mismatch at the ligation junction. Introducing a nucleotide analogue (See Project 3) or mismatched base adjacent to or near the discriminating base may increase the specificity of this reaction. Such modified oligonucleotide primers will be tested in our fidelity assay using wild type and mutant *Tth* ligase. Incorporating modified oligonucleotides in primers to improve the fidelity of *Tth* ligase in discriminating matched from mismatched primers will increase the sensitivity of our PCR/LDR detection of cancer mutations (See Projects 1 and 2).



Our current oligonucleotide design for detecting p53 codon 248 mutations uses the discriminating base on the 3' end to distinguish mutant from wild-type sequence (see Project 2). The specificity of this reaction might be further increased by use of a nucleotide analogue or even mismatched base two or three bases upstream of the 3' terminal base (see Fig. 3). The internal mismatched base or analogue may destabilize the helix and somewhat decrease the efficiency of ligation when the terminal 3' base is perfectly matched. However, one would predict the internal analogue *in addition* to a 3' mismatched base would decrease ligation several orders of magnitude. Destabilizing bases near the 3' end of a primer has already been used to enhance allelic specific PCR amplification [58, 59]. This concept of destabilizing the enzyme-nucleic acid complex to significantly increase the specificity of a reaction is the basis behind our site-specific mutagenesis studies detailed in the preliminary results (i) and methods above (i).

We have extended our ligase fidelity assay to include oligonucleotides containing the **Q<sub>2</sub>** nucleotide analogue (3-Nitropyrole deoxyribonucleoside, See Project 3) adjacent and one base over from the discriminating base (See Fig. ). The **Q<sub>2</sub>** nucleotide analogue was chosen for our initial experiments since it is a "universal" nucleotide analogue, with the ability to base pair to all bases, but without providing hydrogen bonding stability to the analogue-base pair. These oligonucleotides will be synthesized, ligase fidelities assayed as described above, and the results compared with (i) presence of a G:T or C:A mismatch at that position or (ii) perfect complementary at that position (our original oligonucleotides, See Fig. 3). It is possible that destabilization of interactions at or near the ligation junction may enhance the specificity of *Tth* ligase. The effect might be additive; a base mismatch or nucleotide analogue on the common primer may increase fidelity of discrimination of mismatches on the other primer. In addition, increased fidelity may also be achieved by combining modified substrates with use of a mutant *Tth* ligase. Such increased fidelity using a mutant ligase may be specific, demonstrating enhancement only for substrates containing the discriminating and nucleotide analogue base on the 3' side. Alternatively the effect could be global, where enhanced fidelity is observed with the discriminating base on either side. Should this approach be successful, the **Q<sub>2</sub>** nucleotide analogue or mismatched base pair would be incorporated into the appropriate position of the p53 LDR detection oligonucleotides. The fidelity of mutation detection would be compared between the newly designed and original primers.

Nucleotide analogues will help avoid problems in DNA detection caused by polymorphisms surrounding the discriminating nucleotide. Should the target gene contain a natural polymorphism near the mutation in question, the polymorphism may interfere with proper ligation, leading to a false negative signal. The ligase fidelity assay will be used to test the effect of additional mismatches near the discriminating base on both ligation efficiency and fidelity. Again, the **Q<sub>2</sub>** nucleotide analogue will be incorporated into LDR primers at positions of sequence polymorphisms. This "universal" nucleotide analogue will base pair to all bases, while allowing for sequence specific hybridization of the neighboring bases in the primer (See preliminary results, project 3.) Incorporation of the nucleotide analogue inosine into PCR primers allowed for the simultaneous yet specific amplification of *H-ras*, *K-ras*, and *N-ras* gene segments [60]. Based on the preliminary results in Project 3, the **Q<sub>2</sub>** nucleotide analogue should be even more versatile than inosine, especially when base pairing to a guanosine base. This approach should improve the sensitivity of LDR detection for (i) Viral sequences such as HIV and HPV which show considerable polymorphisms (See Project 2) (ii) Drug resistant *M. tuberculosis*, where there are many mutations and third base polymorphisms in the RNA polymerase gene (*rpoB*, Dr. David Persing, Mayo Clinic, personal communication.) (iii) Human genes containing natural polymorphisms nearby disease causing mutations.

**(iii) To determine the three-dimensional structure of *Tth* ligase and of *Tth* ligase/DNA complexes.** *Tth* ligase has been cloned and overproduced to greater than 10% of *E. coli* cellular proteins. Large quantities of the enzyme will be purified for crystallization in the presence and absence of its cofactor nicotinamide adenine dinucleotide (NAD). The crystals will be used to determine the 3-D structure of the ligase by X-ray crystallography. The structure will help provide a structural understanding of its thermostability. The enzyme will also be crystallized in association with a nicked DNA duplex. This structure will help to reveal the mechanism of DNA ligation, and provide an understanding of the specificity of the enzyme towards mismatches at the nicked site. Solving the three dimensional structure of *Tth* ligase will aid in designing new mutants with greater specificity.



The P.I.'s laboratory has successfully overexpressed the *Tth* ligase in *E. Coli*, and purified the enzyme to >98% as judged by staining on an SDS polyacrylamide gel [11]. We have modified the order of the purification scheme so that initial heating of bacterial crude extract to 65°C already purifies the enzyme to 70%. Besides our standard phosphocellulose column, we will also use heparin sepharose [61] or red sepharose which should allow for further purification [62]. Once tens of milligrams of enzyme have been purified to >98%, we will crystallize it both with and without DNA.

(a) *Determination of the structure of Tth ligase.*

(i) *Cocrystallization.* Most of our cocrystallization trials will be by the vapor diffusion method using 1 µl "hanging" drops [63]. This will allow us to screen a large number of conditions with minimal amounts of protein and DNA. The choice of precipitant, pH, counterions, and protein concentration will be routinely varied to determine and optimize the conditions for crystal growth.

We will also attempt to crystallize the enzyme at temperatures exceeding 60°C. Condensation effects limit the usefulness of the vapor diffusion method at these temperatures. Instead, 5 µls of protein-DNA mix will be placed in micro dialysis "buttons" and allowed to equilibrate against the precipitants in small 2 ml vials. These vials can be placed in an oven at high temperatures. For viewing, mounting, and X-ray data collection, the vials will be slowly, over a period of an hour, brought back to room temperature.

In order to crystallize the enzyme with NAD, the cofactor will be incubated with the enzyme for at least half hour prior to crystallization. It may also be possible to soak NAD directly into the enzyme crystals.

Space groups of crystals will be determined by standard precession photography. We will also attempt to freeze the crystals at liquid nitrogen temperatures in order to minimize radiation damage.

(ii) *Data Collection.* The optimal choice for the collection of X-ray data, at present, is image plates with synchrotron radiation [64]. The accuracy and large dynamic range of image plates combined with the high intensity of synchrotron radiation yields probably the best data that can be attained from any given crystal. Many synchrotrons also possess a "freezing" setup that essentially eliminates the radiation damage problem for many crystals. Several synchrotron facilities have successfully installed an imaging plate system, including the Photon Factory (line BL6A2, Tsukuba, Japan) and Cornell High Energy Synchrotron Source (CHESS, lines F1 and F2), and Brookhaven. Data collected at the Photon factory on the Weissenberg camera will be processed by the WEIS program package [65], already installed on a Silicon Graphics machine in our laboratory. Any data collected at CHESS or Brookhaven will be processed by the DENZO program package [66] already installed on a VAX workstation at Columbia.

Although synchrotron radiation is the optimal choice for data collection, it is by no means the most practical or convenient. Area detectors installed on rotating anodes are by far the most convenient mode for data collection. As a general scheme, we will aim to collect data from our crystals on the Xuong-Hamlin detector [67], installed in the Columbia University diffraction facility. The merging of the data and the calculation of statistics will be carried out by programs AGROVATA and ROTAVATA belonging to the CCP4 suite of programs [68].

(iii). *Heavy Atom Derivatives.* For protein crystals, heavy atom derivatives can be found by the standard methods of soaking the crystals in different heavy atom solutions, at different concentrations, and different lengths of time. Since the protein is expressed in a bacterial system, it is also possible to prepare derivatives by substituting methionine residues for selenomethionines [69].

(iv). *Phasing.* The positions of heavy atoms will be found by inspection of a difference Patterson maps as well as by automated Patterson search methods. The positions will be refined by lack of closure or origin removed Patterson methods as implemented in the program HEAVY [70]. The phases from these heavy atom sites will then be used to locate any remaining sites by difference Fourier methods. The phases from all the heavy atom sites will be used to compute electron density maps. Chain tracing and other forms of electron density fitting will be carried out on a IRIS graphics workstation with the aid of program package O [71]. Any comparisons of structures will also be carried out on the IRIS workstation.

In parallel with the above heavy atom method, we will also pursue the MAD method [53]. Multiwavelength data collected from either a heavy atom derivative or a selenomethionine variant of the protein will be processed by the MADSYS program package. Phases will be derived by the application of MADSYS [72] and MLPHARE [73] programs to the processed data.

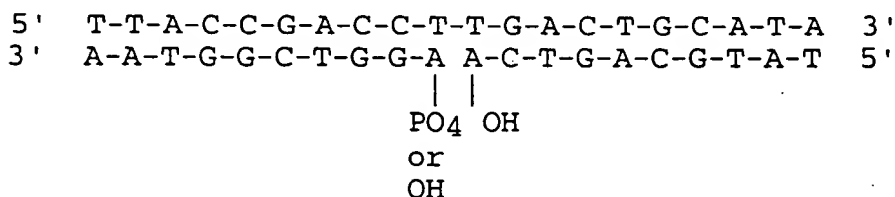
(b) *The structure of Tth ligase complexed with DNA.*

(i) *Determinating factors affecting complex formation between Tth ligase and DNA substrate.* We will identify conditions under which *Tth* ligase can bind DNA, in the absence of its joining activity. Using standard gel shift procedures [74], we will first examine the effect of omitting NAD and divalent cations on complex formation. NAD and divalent cations such as  $Mg^{2+}$  are essential for the catalytic activity, but their effect on complex formation have not yet been investigated. We will also synthesize a DNA substrate lacking a phosphoryl group at the 5' end of the nicked site and determine again by gel shifts whether a protein-DNA complex can still be formed. The effect of replacing the 5' phosphoryl group with thio and phosphonate analogs as well as replacing 3' nucleotide with F-ara-AMP or 6-thio-GMP on DNA binding will also be examined [75, 76]. The monovalent cation  $Na^+$  was shown to completely inhibit the *T. thermophilus* HB8 ligase at concentrations of 0.15M [77] human DNA ligases I and II at concentrations of 0.8 mM [78]. The catalytic activity of *Tth* ligase will be determined at different monovalent and divalent cation concentrations, and gel shifts performed at inhibitory concentrations. Alternatively, we will use one of our catalytically defective mutant, such as K118H, K118L or D120A in the gel shift assays.

For co-crystallization experiments, it would also be helpful to know the minimal length of DNA that can bind *Tth* ligase. Once we have identified conditions for a stable enzyme-DNA complex, we can determine the extent of nicked DNA substrate protected by hydroxyl radical footprinting [79]. Alternatively, we will synthesize a series of DNA substrates that range in size from 10 to 30 bps, and perform gel-shift experiments at different protein concentrations.

These experiments should identify conditions and/or mutant enzymes which allow for effective formation of the enzyme DNA complex in the absence of catalysis.

(ii) *Cocrystallization.* Many of the principles and methods in the cocrystallization of DNA binding proteins with oligodeoxynucleotides have been discussed by the Co-investigator [80]. The most important factors that affect cocrystallization are the length of the DNA fragment and the nature of the terminal nucleotides [81]. Once we have established the conditions for a stable ligase-DNA complex, we will synthesize a series of DNA fragments that extend the "minimal" site by 1-bp. Oligonucleotides will be prepared with both overhanging and blunt ends. We will also vary the bases at the ends of our oligomers. DNA fragments with a nicked site will require the synthesis and hybridization of three DNA strands, as shown below for a hypothetical blunt ended 20 mer:



(iii) *Data Collection.* Similar considerations as described above for the *Tth* ligase enzyme in the absence of DNA substrate.

(iv) *Heavy Atom Derivatives.* For protein-DNA complexes, derivatives are most easily prepared by substituting 5-bromouracil or 5-iodouracil for thymine bases on the DNA. Synthesis of DNA with iodouracils requires several precautions, such as keeping the reagents in the dark, reducing the time of treatment with ammonium hydroxide, increasing coupling times etc. Many of these precautions and methods have already been implemented in our laboratory.

(v) *Phasing*. The positions of heavy atoms, determined from Patterson maps, will be refined by program MLPHARE [73]. The phases from the refined sites will be used to calculate an electron density map and a three dimensional built with the aid of program O. If we manage to determine the structure of *Tth* ligase by itself, we will construct a Molecular Replacement (MR) model from the protein and use it to determine the rotational and translational parameters. These parameters will be further refined with the aid of program CORELS [82], and a set of MR phases calculated. These phases will be combined with any heavy atom phases and a combined electron density map calculated.

The structure of *Tth* ligase will help to reveal the mechanism of DNA ligation, and provide an understanding of the sensitivity of the enzyme towards mismatches at the nicked site. The structure will reveal several amino acid residues which interact with the sugar-phosphate backbone of DNA. These residues would be prime candidates for further site-specific mutagenesis. Solution of the three dimensional structure of *Tth* ligase will aid in the design of new mutants with potentially greater specificity.

## E. PROGRAM ASPECTS

We are developing a comprehensive approach to understanding the mechanism of *Tth* ligase and improving its fidelity for discriminating perfectly matched from mismatched substrates. The three parts to this program are: (i) Developing a rapid assay to test different reaction conditions, mutant *Tth* ligases, and modified DNA substrates for their effect on the fidelity of ligase. (ii) Determining the 3-dimensional structure of *Tth* ligase-DNA complex. (iii) Site specific mutagenesis based on protein sequence homology and protein-sugar-phosphate backbone contacts as determined from the X-ray structure.

These studies should lead to conditions which increase the specificity of *Tth* ligase. Improving the fidelity of *Tth* ligase in discriminating matched from mismatched primers will increase the sensitivity of our PCR/LDR detection of cancer mutations (See Projects 1 and 2). Incorporating nucleotide analogues (Project 3) in the primers may further enhance *Tth* ligase fidelity. For example, we are currently able to identify the presence of a perfectly matched template (cancer mutation) that is just 1% of the mismatched template (normal cells). Improving the fidelity of this reaction could allow us to detect 1 cancer cell in  $10^2$  to  $10^3$  normal cells. The PCR /LDR format would allow for multiplex detection of many different mutations in a gene at this high level of sensitivity.

A *Tth* ligase with improved specificity would be of benefit to several of our collaborators. This will aid in detection and identification of: antibiotic resistant and slow growing bacterial pathogens (Dr. Carl Batt, Dr. Patrice Courvalin), multiple drug resistant *M. tuberculosis* (Dr. David Persing), viral pathogens such as HIV, and high risk HPV strains (Dr. Olen Kew and Dr. Saul Silverstein), multiple germline mutations in single gene disorders (Dr. Vicky Herrera, Dr. Eric Hoffman, Dr. Perry White, and Dr. Emily Winn-Deen), and multiple somatic mutations in tumor suppressor genes and oncogenes (Dr. John Kovach, Dr. Michael Osborne, Dr. Basil Rigas, Dr. John Sninsky, Dr. Mark Sobel, Dr. Steven Sommer, and Dr. Thierry Soussi). Please see letters of collaboration in the overview section of this program project grant.

## F. TIMETABLE

### Task 1. Constructing mutant thermostable ligases and testing for greater specificity.

- a. Optimizing the rapid assay to test the fidelity of wild type and mutant *Tth* ligases. Varying the pH, salt, temperature, oligonucleotide concentrations, LDR conditions, etc. of our ligase fidelity assay to obtain maximum discrimination between matched and mismatched substrates. Months 1-18.
- b. Characterizing mutant *Tth* ligases already isolated using the above assay. We aim to increase the sensitivity of this assay to detect 1 cancer gene mutation in  $10^2$  to  $10^3$  normal cells. Months 12-36.
- c. Isolating and characterizing additional mutant *Tth* ligases. Construct and assay ligase genes altered at conserved arginine and lysine residues. Determining the three-dimensional structure of *Tth* ligase, and of *Tth* ligase/DNA complexes will aid significantly in the design of new mutants. Months 24-60

**Task 2. Testing modified oligonucleotides for greater specificity during ligation.**

- a. Synthesizing oligonucleotides containing a nucleotide analogue (See Project 3) or mismatched base adjacent to or near the discriminating base of an LDR primer. Months 1-12.
- b. Testing modified oligonucleotide primers in our fidelity assay using wild type and mutant *Tth* ligase. Months 6-30.
- c. Incorporating modified oligonucleotides into p53 primers to improve the fidelity of *Tth* ligase in discriminating matched from mismatched primers. Testing whether modified oligonucleotides increase the sensitivity of our PCR/LDR detection of cancer mutations (See Projects 1 and 2). Months 18-60.

**Task 3. Determining the three-dimensional structure of *Tth* ligase and of *Tth* ligase/DNA complexes.**

- a. Purifying large quantities of *Tth* ligase for crystallization. Months 1-12.
- b. Testing co-crystallization conditions for *Tth* ligase in the presence and absence of its cofactor nicotinamide adenine dinucleotide (NAD). Months 6-18.
- c. Determining the 3-D structure of *Tth* ligase by X-ray crystallography. Months 18-60.
- d. Crystallizing *Tth* ligase in association with a nicked DNA duplex.
- e. Determining the 3-D structure of *Tth* ligase complexed to nicked DNA by X-ray crystallography. Solving the three dimensional structure of *Tth* ligase will aid in designing new mutants with greater specificity. Months 18-60.

**G. HUMAN SUBJECTS / VERTEBRATE ANIMALS:** Not applicable

**I. CONSULTANTS/COLLABORATORS:** Letters and Biographical Sketches for collaborators are attached in the overview section of this program project grant.

**J. CONSORTIUM/CONTRACTUAL ARRANGEMENTS:** Please see following page.



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STATEMENT OF INTENT TO ESTABLISH A CONSORTIUM AGREEMENT

Date: January 26, 1994

Grant Number: P01-

P-01 Application Title: PROGRAM PROJECT: NEW METHODS FOR  
CANCER DETECTION

Project # 4; ENGINEERING AN IMPROVED  
THERMOSTABLE LIGASE.

Proposed Project Period: Year 01; 12/01/94- 11/30/95

"The appropriate programmatic and administrative personnel of each institution involved in this grant application are aware of the NIH consortium grant policy and will establish the necessary inter-institutional agreement(s) consistent with that policy."

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**K. LITERATURE CITED.**

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## **Project 5.**

### **Design and Synthesis of DNA and PNA Arrays**

**Project Leader: George Barany**  
**University of Minnesota**

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. **DO NOT EXCEED THE SPACE PROVIDED.**

The goal of this program project is to develop methods for identifying multiple gene mutations in cancers. For maximum utility, these methods must be able to recognize and discriminate between dozens or hundreds of mutations.

To accomplish this, we propose to capture specific ligase detection reaction (LDR) products on a spatially addressable array, such that the position of a signal identifies a mutation. Each LDR product will have a "zip code" tail, which will be selectively captured by a "complementary zip code" on a solid support. The complementary components can be DNA oligonucleotides or peptide nucleotide analogues (PNA). PNA/DNA hybrids have significantly higher  $T_m$  values than DNA/DNA hybrids. Incorporation of the nucleotide analogue, 5-propynyluridine, into DNA zip code and PNA address sequences will further increase and optimize  $T_m$  values (Project 3). Unreacted LDR primer may therefore be washed away at high temperatures allowing for a higher sensitivity in detecting LDR products. A reusable, universal addressable array could be used for detecting a wide range of cancer mutations, genetic diseases and infectious agents.

Implementation of these concepts, with the ultimate goal of achieving reliable and efficient materials and procedures that can be incorporated into easy-to-use, automated, low-cost diagnostic devices, will follow these aims: (i) Development and evaluation of solid support materials compatible with chemical synthesis of DNA oligonucleotides and PNA oligomers, and compatible with subsequent hybridization reactions. Surfaces, beads, or membranes will be functionalized, and extended as needed with hydrophilic spacers such as heterobifunctional polyethylene glycol (PEG) and/or carbohydrates. Chemistry for linking oligomers to the solid support, and/or solid-phase assembly of oligomers, will be developed. (ii) Establishment of methodology for synthesis of spatially addressable arrays of DNA oligonucleotides and PNA oligomers. Appropriate masking technology will expose defined regions of the solid support for attachment of pre-formed oligomers, or for chain elongation to assemble the needed oligomers. In the latter mode, segment condensation will be used when possible in order to provide efficient convergent synthesis, and because chemical "failures" will become "invisible" during the subsequent hybridization. (iii) Demonstration of scope and limitations of zip code concepts. As aims (i) and (ii) come to fruition, testing will be carried out (Core B). Design of primer and zip code structures will be facilitated by the informatics collaboration (Core A).

PERSONNEL ENGAGED ON PROJECT, INCLUDING CONSULTANTS/COLLABORATORS. Use continuation pages as needed to provide the required information in the format shown below on *all* individuals participating in the project.

Name	BARANY, George	Degree(s)	Ph.D.	Social Security #	REDACTED
Position Title	Professor	D.O.B.	REDACTED	Role on Project	Prin. Investig.
Organization	University of Minnesota			Department	Chemistry
Name	HAMMER, Robert	Degree(s)	Ph.D.	Social Security #	REDACTED
Position Title	Assistant Professor	D.O.B.	REDACTED	Role on Project	Co-investigator
Organization	Louisiana State University			Department	Chemistry
Name	VAGNER, Josef	Degree(s)	Ph.D.	Social Security #	REDACTED
Position Title	Postdoctoral Associate	D.O.B.	REDACTED	Role on Project	
Organization	University of Minnesota			Department	Chemistry
Name	VAGNEROVA, Lydie	Degree(s)	B.S.	Social Security #	pending
Position Title	Research Technician	D.O.B.	REDACTED	Role on Project	
Organization	University of Minnesota			Department	Chemistry
Name		Degree(s)		Social Security #	
Position Title		D.O.B.		Role on Project	
Organization				Department	
Name		Degree(s)		Social Security #	
Position Title		D.O.B.		Role on Project	
Organization				Department	

DD

Principal Investigator/Program Director (Last, first, middle):  
**DETAILED BUDGET FOR INITIAL BUDGET PERIOD**  
**DIRECT COSTS ONLY**

F. BARANY, Ph.D.

FROM

94/12/01

THROUGH

95/11/30

PERSONNEL (Applicant Organization Only)					DOLLAR AMOUNT REQUESTED (omit cents)		
NAME	ROLE ON PROJECT	TYPE APPT. (months)	% EFFORT ON PROJ.	INST. BASE SALARY	SALARY REQUESTED	FRINGE BENEFITS	TOTALS
George Barany (AY)	Principal Investigator	9	5				
George Barany (SS)	Principal Investigator	3	16.7				
Josef Vagner	Post-Doc Associate	12	100				
Lydie Vagnerova	Research Technician	12	100				
<b>PROJECT 5</b>							
SUBTOTALS					\$55,623	\$8,446	\$64,069
CONSULTANT COSTS							
EQUIPMENT (Itemize)							\$0
SUPPLIES (Itemize by category)							
Chromatography \$3,000							
Chemicals \$5,000							
Special Solvents & Reag for PNA synthesis \$7,500							\$15,500
TRAVEL							
One trip per year for P.I. to present results \$1,200							\$1,200
PATIENT CARE COSTS		INPATIENT					\$0
		OUTPATIENT					\$0
ALTERATIONS AND RENOVATIONS (Itemize by category)							\$0
OTHER EXPENSES (Itemize by category)							
See following page \$5,000							\$5,000
<b>SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD</b>							<b>\$85,769</b>
CONSORTIUM/CONTRACTUAL COSTS							
DIRECT COSTS						TOTAL	\$34,308
INDIRECT COSTS 40% Direct							
<b>TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD</b>							<b>\$120,077</b>

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(Form Page 4) Page

Number pages consecutively at the bottom throughout the application. Do not use suffixes such

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DD

**BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD  
DIRECT COSTS ONLY**

PROJECT 5

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED			
			2nd	3rd	4th	5th
<b>PERSONNEL:</b>						
Salary & fringe benefits						
Applicant organization only		\$64,069	\$66,632	\$69,297	\$72,069	\$74,952
<b>CONSULTANT COSTS</b>		\$0	\$0	\$0	\$0	\$0
<b>EQUIPMENT</b>		\$0	\$2,000	\$2,000	\$2,000	\$2,000
<b>SUPPLIES</b>		\$15,500	\$16,120	\$16,765	\$17,436	\$18,133
<b>TRAVEL</b>		\$1,200	\$1,248	\$1,298	\$1,350	\$1,404
<b>PATIENT CARE COSTS</b>	<b>INPATIENT</b>	\$0	\$0	\$0	\$0	\$0
	<b>OUTPATIENT</b>	\$0	\$0	\$0	\$0	\$0
<b>ALTERATIONS AND RENOVATIONS</b>		\$0	\$0	\$0	\$0	\$0
<b>OTHER EXPENSES</b>		\$5,000	\$5,200	\$5,408	\$5,624	\$5,849
<b>SUBTOTAL DIRECT COSTS</b>		\$85,769	\$91,200	\$94,768	\$98,479	\$102,338
<b>CONSORTIUM/ CONTRACTUAL COSTS</b>		\$34,308	\$35,680	\$37,107	\$38,591	\$40,135
<b>TOTAL DIRECT COSTS</b>		\$120,077	\$126,880	\$131,875	\$137,070	\$142,473
<b>TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD</b>						<b>\$658,375</b>

(Item 8a)-&gt;

**JUSTIFICATION** (Use continuation pages if necessary):

**From Budget for Initial Period:** Describe the specific functions of the personnel, collaborators, and consultants and identify individuals with appointments that are less than full time for a specific period of the year, including VA appointments.

**For All Years:** Explain and justify purchase of major equipment, unusual supplies requests, patient care costs, alterations and renovations, tuition remission, and donor/volunteer costs.

**From Budget for Entire Period:** Identify with an asterisk (\*) on this page and justify any significant increase or decrease in any category over the initial budget period. Describe any change in effort of personnel.

**For Competing Continuation Applications:** Justify any significant increases or decreases in any category over the current level of support.

**INITIAL BUDGET PERIOD:**

**General:** This budget covers only the expenses in Dr. George Barany's laboratory at the University of Minnesota, and represents modest levels by comparison to other ongoing and past grants from NIH. The significant intellectual and experimental contributions to the project of Dr. Robert P. Hammer from Louisiana State University, and Dr. Francis Barany of Cornell University Medical College, are supported by separate budgets in this program project.

**Personnel:** The preparation, manipulation, and characterization of oligonucleotide and PNA building blocks and oligomers, as well as of a range of solid supports needed for synthesis and hybridizations, is quite labor-intensive and requires experienced and well-trained co-workers. Dr. Josef Vágner is a highly qualified peptide chemist who has been working in Dr. Barany's laboratory for over a

year on other projects. Ms. Vágnerova holds a degree in biochemistry and has recently arrived in this country to join the research group as a technician. These two individuals will be able to make an immediate impact to this Research Plan. Salaries follow University of Minnesota guidelines, and fringe benefits are calculated at 23.7% academic, 3.6% postdoctoral, and 27.5% civil service. Professors have 9-month academic appointments, and need to cover the 3-month summer salary from grants.

*Equipment:* Dr. Barany's laboratory has all of the major instrumentation required to carry out this research, with several new instruments acquired recently to replace and/or augment older models (listed with "Resources and Environment").

*Supplies and Other Expenses:* We are perpetually underfunded in these categories, and require a combination of grants to cover these costs. "Supplies" include chemical reagents, consumable supplies, and chromatography expenses. "Other Expenses" cover analytical fees (NMR, mass spectrometry, elemental analysis), instrument maintenance (service contracts on peptide synthesizers and amino acid analyzer shared with other grants), publication costs, communications, etc.

*Travel:* Funds are requested to allow attendance at one professional meeting per year in order to present results and learn of advances in scientific fields related to this proposal.

*Consortium/Contractual Costs:* The University of Minnesota negotiated (May 13, 1992) indirect cost rate is 40% of modified direct costs (total direct costs - equipment - graduate student benefits).

#### CONTINUATION YEARS:

*Personnel:* Dr. Barany's research program attracts a constant stream of postdoctoral applicants from good laboratories throughout the world, so there will be no difficulty in appointing individuals to continue the work after Dr. Vágner leaves. Similarly there is a good pool of candidates for technician positions. The "Research Plan" will require constant staffing at the level of two individuals.

*Equipment:* We request \$2,000 per year to cover relatively small items of lab hardware.

*Increases:* Following NIH guidelines, the percentage recurring annual increase in all costs are calculated at 4%.

Five years of support are requested in order to allow enough time to show significant progress on the goals of the "Research Plan."



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**RESOURCES AND ENVIRONMENT**

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**FACILITIES:** Mark the facilities to be used at each performance site listed in Item 9, Face Page, and briefly indicate their capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Use "Other" to describe the facilities at any other performance sites listed in Item 9 on the Face Page and at sites for field studies. Use continuation pages if necessary. Include an explanation of any consortium/contractual arrangements with other organizations.

- ☐ **Laboratory:** The Barany group currently numbers about a dozen full-time researchers, and two and a half 490 ft<sup>2</sup> laboratories (Kolthoff 476, 468, and 470; listed in order of length of occupancy; 4 desks per lab; common service corridor). These labs are down the hall from the faculty office. A 260 ft<sup>2</sup> laboratory (Kolthoff 463A, 1 desk) adjoins the office and is used for work by Dr. Barany and a laboratory technician and /or undergraduate research assistants. The group also occupies a 300 ft<sup>2</sup> instrumentation room (Kolthoff 472), and has a 125 ft<sup>2</sup> section of a shared instrumentation room (498A). All of this is in the Department of Chemistry on the Minneapolis campus of the University of Minnesota.
- ☐ **Clinical:**
- ☐ **Animal:**
- ☐ **Computer:** Three Macintosh personal computers for word processing and graphics
- ☐ **Office:** 140 ft<sup>2</sup> (Kolthoff 461)
- ☐ **Other ( ):**

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**MAJOR EQUIPMENT:** List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

MilliGen/Biosearch 9050 and 9600 Peptide Synthesizers (acquired 1990); Beckman System 6300 High Performance Amino Acid Analyzer (acquired 1989); Beckman-Altex analytical gradient HPLC apparatus (acquired 1981) with variable wavelength UV detector and Hewlett-Packard recording integrator; Waters Delta-Prep 3000 HPLC (acquired 1988) apparatus with UV detector, integrator, and automatic sample injector; Beckman P/ACE 2100 capillary zone electrophoresis system (acquired 1991); MPLC set-up; UV-visible spectrophotometer; photolysis equipment; fraction collectors; Labconco lyophilizer; basic organic synthesis set-up; excellent hoods in all of the laboratories.

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**ADDITIONAL INFORMATION:** Provide any other information describing the environment for the project. Identify support services such as consultant, secretarial, machine shop, and electronics shop, and the extent to which they will be available to the project.

Buildings of the Department of Chemistry contain major instrumental facilities for routine and high-field <sup>1</sup>H and <sup>13</sup>C-NMR, IR, mass spectrometry (including FABMS), and X-ray diffraction which are extensively used in this research program. Machine, electronics, and glassblowing shops are on-site, as is a research stockroom. The Department offers quarter-time secretarial support to the faculty, and Dr. Barany occasionally gets additional secretarial help paid for by research grants. An additional resource for this research is the Microchemical Facility of the University of Minnesota Institute of Human Genetics on the Minneapolis campus, which includes state-of-the-art equipment for amino acid analysis, peptide sequencing and synthesis, oligonucleotide synthesis, and other procedures.

## A. SPECIFIC AIMS:

This Research Plan seeks to develop and optimize new tools that will be essential components to the integrated approach to cancer, genetic, and infectious disease detection described in the overall program project application. A novel polymerase chain reaction/ligase detection reaction (PCR/LDR) method for discriminating normal, carrier, and disease individuals has been described, and a high-sensitivity PCR/restriction endonuclease/LDR (PCR/RE/LDR) method for detection of rare cancer mutations is under development (Projects 1 and 2). These technologies, in their present form or as improved by applications of "convertide" nucleotide analogue bases (Project 3) and/or engineered thermostable ligase (Project 4), will be carried out in multiplex formats to simultaneously identify many mutations. LDR products, derived from one fluorescent primer and an adjacent primer with extra nucleotides or hexaethylene oxide "tails", are currently separated by size on an automated DNA sequencer, or by capillary electrophoresis. Use of different fluorescent groups allows a second dimension of mutation discrimination.

Herein, we propose new solid-phase approaches for simultaneous detection of multiplex LDR products. The general idea is that specific products will be captured on a spatially addressable array, so that the position of a signal identifies a mutation. Each LDR product will have a "zip code" tail, which will be captured selectively by a "complementary zip code" on the solid support. The supported (complementary) components can be modified DNA oligonucleotides or peptide nucleotide analogues (PNA), designed so that the resultant zip code hybrids have a significantly higher  $T_m$  than DNA/DNA hybrids. Unreacted primers may be washed away at high temperatures, allowing for detection of the LDR product. Multiple reuse of a universal "complementary zip code" array is envisaged to allow detection of a wide range of cancers and genetic diseases.

Implementation of these concepts, with the ultimate goal of achieving reliable and efficient materials and procedures that can be incorporated into easy-to-use, automated, low-cost diagnostic devices, will follow these aims:

**(i) Development and evaluation of solid support materials compatible with chemical synthesis of DNA oligonucleotides and PNA oligomers, and compatible with subsequent hybridization reactions.** Both commercially available and experimental materials will be screened. Surfaces, beads, or membranes will be functionalized, and extended as needed with hydrophilic spacers such as heterobifunctional polyethylene glycol (PEG) and/or carbohydrates. Chemistry for linking oligomers to the solid support, and/or solid-phase assembly of oligomers, will be developed.

**(ii) Establishment of methodology for synthesis of spatially addressable arrays of DNA oligonucleotides and PNA oligomers.** Appropriate masking technology will expose defined regions of the solid support for attachment of pre-formed oligomers, or for chain elongation to assemble the needed oligomers. In the latter mode, segment condensation will be used when possible in order to provide efficient convergent synthesis, and because chemical "failures" will become "invisible" during the subsequent hybridization.

**(iii) Demonstration of scope and limitations of zip code concepts.** As aims (i) and (ii) come to fruition, testing will be carried out (Core B). Design of primer and zip code structures will be facilitated by the informatics collaboration (Core A).

## B. BACKGROUND AND SIGNIFICANCE

The cancer-detection technology of this program project application relies in good part on the capability to prepare by rapid, accurate chemical methods a multitude of oligonucleotide and related structures of defined sequence in the 20 to 50-base size range. The revolutionary solid-phase approach, introduced by Merrifield in the 1960's for peptides, points the way and provides ample experiences and precedents [1-6]. In solid-phase methodology that has been refined substantially and is readily automated, suitably protected amino acid building blocks are added in order (C to N) to a growing chain which is attached covalently through the C-terminus to a polymeric support. The principal commercially available

chemistries are referred to as "Boc" and "Fmoc", abbreviations for the names of the key *temporary* protecting groups. Often, a *linker* or *handle* is used to mediate the initial anchoring. Reactions are driven to completion by the use of excess reagents, which are removed by simple filtration and washing steps; the chemistry can also be carried out in columns in a continuous-flow mode. Upon completion of chain assembly, protecting groups are cleaved and the peptide is released into solution for further purification and characterization. An important aspect for successful results is the choice of the polymeric support. For many years, most work was carried out on 1% cross-linked microporous polystyrene resins (beaded), or on polyacrylamides (these latter could be embedded within an inorganic matrix, e.g., kieselguhr, or a rigid macroporous polystyrene, e.g., Polyhipe) [6-12]. Within the past few years, several additional materials with interesting physico-chemical properties have become available from several academic laboratories and commercial sources. These include membranes [13, 14], cotton and other appropriate carbohydrates [15-18], controlled-pore silica glass [19], and linear polystyrene grafted onto Kel-F [20]. A particularly interesting concept involves the use of polyethylene glycol-polystyrene supports (PEG-PS or Tentagel), which are compatible with both batchwise and continuous-flow reactors, and may facilitate difficult chemistries in peptide synthesis [21-23]. Other recent trends from the peptide field which are relevant to the goals of this proposal involve synthesis on polymeric surfaces, and the simultaneous preparation of multiple structures. In these procedures, due to Geysen, Frank, and research groups at Affymax, Arris, and Millipore, among others, relatively short peptides are built up on appropriately modified polyethylene pins, cellulose or polypropylene membranes, or glass surfaces, in a way that the bound final structures (purities in the 60 to 90% range) are tested directly by ELISA or other biological testing methods [15, 24-27]. The active structures are then deduced from their physical position, i.e., *spatial address* on the array. In an alternative combinatorial *library* approach developed at Selectide [28], millions of peptides are generated simultaneously by successive cycles of randomization/remixing of beaded supports. Intrinsic to the design of these experiments, each individual bead contains only a single peptide, so that those beads giving a positive interaction with a receptor can be picked out and subjected to analytical procedures that give the structure on the bead.

Solid-phase oligonucleotide synthesis has come to the fore in the past eight years with the development of reliable high-efficiency phosphoramidite [29] or H-phosphonate chemistry [30, 31] for linking protected nucleoside building blocks. Synthesis (3' to 5') is usually supported on controlled-pore glass, although other materials can also serve. The current automated methodology can routinely furnish oligonucleotides of length > 50 residues in overall purity > 90% directly upon release from the support. The methodology can also be adapted to incorporate unusual nucleotide bases, as well as modifications in the phosphodiester backbone (e.g., non-bridging thio or dithio substitution) and end-group labels (e.g., fluorescent dyes, biotin) [32]. As with peptides, relatively short oligonucleotides can be synthesized in spatially addressable arrays on glass surfaces [33-37]. Alternatively, a variety of procedures have been described for site-specific attachment of pre-synthesized oligonucleotide probes to nylon membranes or inside polyacrylamide gels [38-40]. Such arrays have been applied for DNA hybridization reactions, with applications to DNA sequencing or detection of biotinylated PCR-amplified products. These earlier studies provide useful precedents to some of the goals of this proposal.

Within the last few years, a group from Denmark [41-44] has introduced novel peptide nucleotide analogue (PNA) oligomers which mimic closely the spatial arrangement of the oligonucleotide backbone, but use nonchiral (2-aminoethyl)glycine units to replace the sugar phosphodiester (Figure 1). Additional innovations for PNA chemistry are under development at Millipore, in close consultation and collaboration with us [45]. These improvements include complete protection schemes for all the "bases" in concert with Boc, Fmoc, or alternative chemistries, optimized coupling (note: since racemization is not an issue, strong activation methods can be applied) and capping protocols, and efficient sequencing procedures. PNA and single-stranded DNA join to form anti-parallel heteroduplexes that exhibit Watson-Crick specificity and (particularly under low-salt conditions) tighter binding (higher  $T_m$ ) than the corresponding double-stranded DNA [44]. As is discussed later, these properties dovetail extremely well with some of the requirements for the multiplex cancer detection protocol proposed in this program project application.

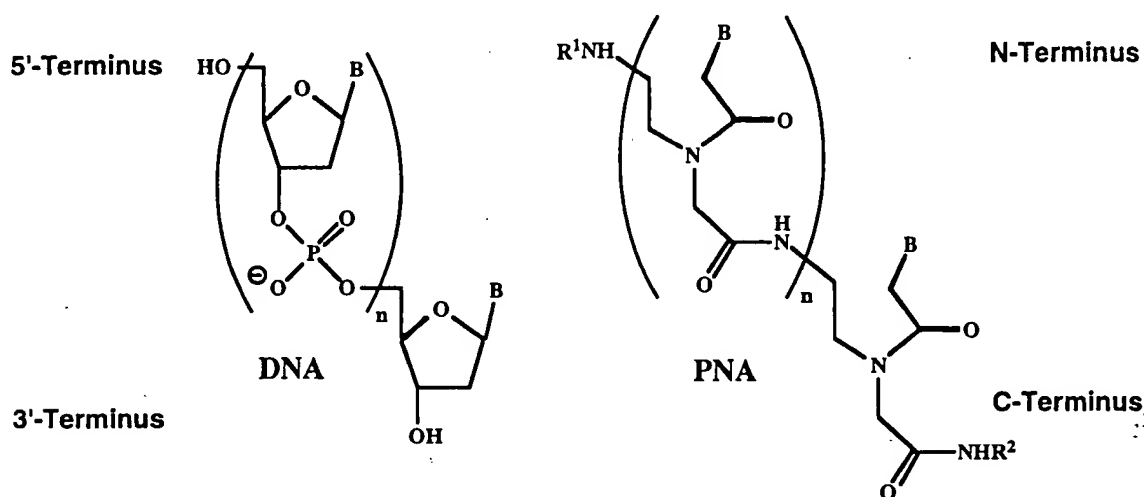


Fig. 1. Structural similarity of DNA and PNA.

### C. PRELIMINARY STUDIES

The previous section of this proposal gave a brief overview of the current status of solid-phase methodologies, with an emphasis on general aspects which are relevant to the objectives of the research program. The following paragraphs provide brief descriptions of recent experimental advances from our laboratories which place us at the cutting edge of methodological developments.

We have invented several procedures for grafting both homo- and heterobifunctional polyethylene glycol (PEG) derivatives of defined molecular weight onto amino-functionalized polystyrene (PS); the resultant microporous beaded PEG-PS supports are now commercially available through Millipore and have numerous advantageous properties with respect to polystyrene [22, 23]. Our extensive experience in this field will be needed to introduce PEG as a "spacer" separating oligonucleotide or PNA molecules from functionalized surfaces. A useful start in this regard was reported recently by our collaborators Dr. Dereck Hudson and Dr. Ronald Cook [27] who in turn derivatized polyethylene plates, modified the resultant surfaces with PEG, and coupled carboxymethyl dextran to impart further hydrophilicity and serve as a starting point for peptide synthesis. (Please see letters of collaboration in overview section of program project).

PEG-PS has proven to be an ideal support for peptide library studies by the Selectide process [28]. PEG-PS is compatible with the organic reagents and solvents for efficient synthesis throughout the beads, and also has sufficient hydrophilic character to allow biological testing in aqueous milieus. Based on the realization that biological interactions occur only at the surface of beads, we have devised methodology for differentiation of "surface" and "interior" areas. PEG-PS is loaded with Boc-Trp-Gly, following which chymotryptic digestion "shaves" selectively only substrates at the surface which are accessible to the macromolecular enzyme. The exposed glycine is the starting point for orthogonal peptide synthesis using Fmoc chemistry, so that each bead is charged with a "screening" peptide at the surface, representing <1% of the total content but responsible for the entire spectrum of biological interaction. In concert, Boc chemistry establishes a sequenceable "coding" peptide confined to the interior, representing the vast majority of material on the bead but restricted from biological interaction [46]. This "shaving" concept has implications beyond the application to encoded combinatorial libraries just described; in the context of the present Research Plan, it can be used to ensure that oligopolymers synthesized on surfaces will be able to hybridize to oligonucleotides.

Our laboratories have also pioneered the development of novel linkers and handles for peptide synthesis [47, 48]; the extension to DNA and PNA is expected to be straightforward. Of particular interest, we have developed tris(alkoxy)benzyl amide (PAL) [49] and ester (HAL) [50] linkages, which upon cleavage with acid provide respectively C-terminal peptide amides, and *protected* peptide acids that can be

used as building blocks for so-called *segment condensation* approaches. We have noticed that the stabilized carbonium ion generated in acid from cleavage of PAL or HAL linkages can be intercepted by tryptophanyl-peptides. While this reaction is a nuisance for peptide synthesis and preventable (in part) by use of appropriate scavengers, we envisage herein a positive application to chemically "capture" oligo-Trp-end-labelled DNA and PNA molecules by HAL-modified surfaces (see Fig. 7 in Experimental Designs and Methods).

## D. EXPERIMENTAL DESIGN AND METHODS

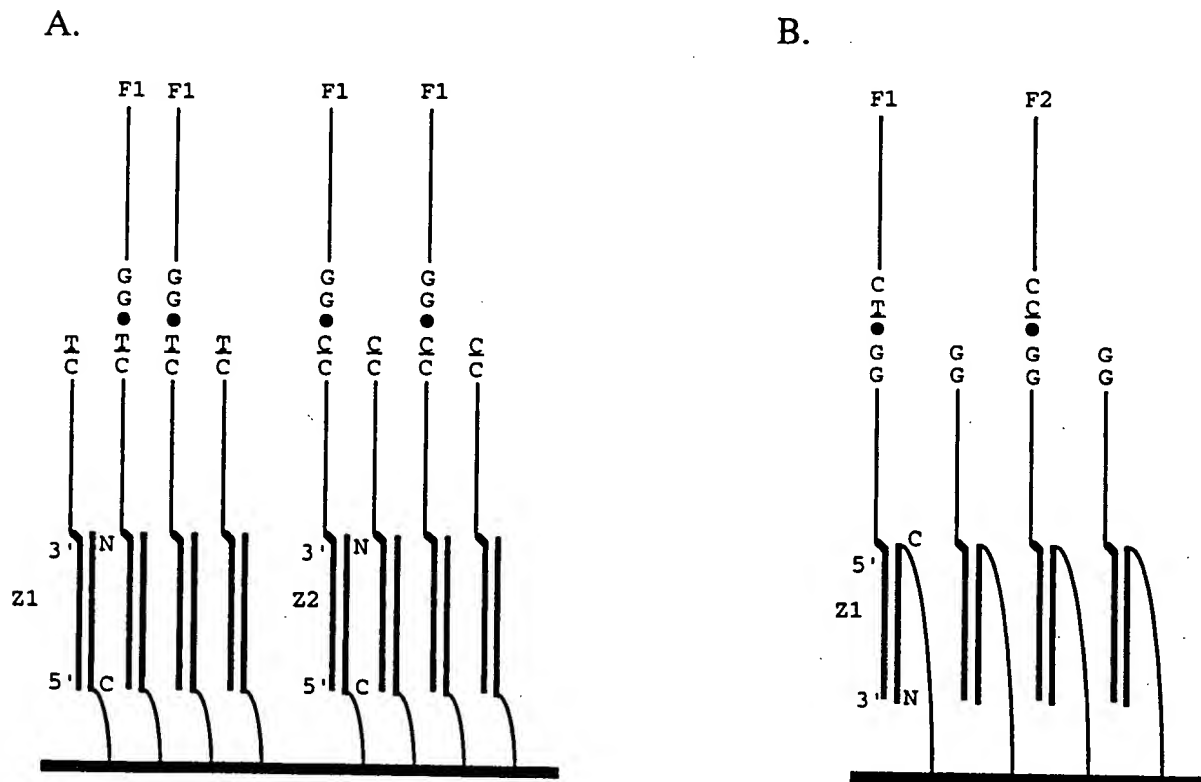
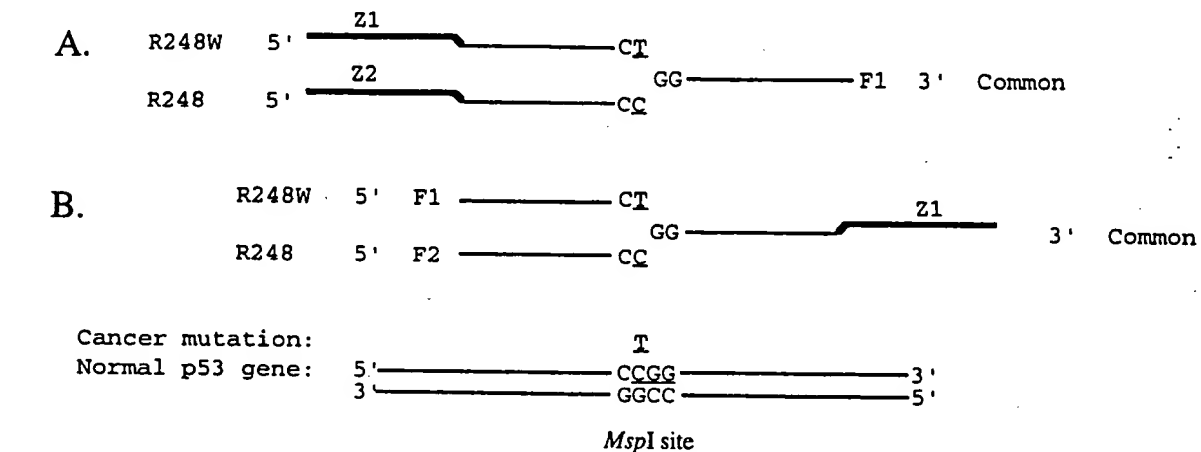
### (i) Overview

(a) *Array technology.* This Research Plan describes a systematic approach to the design and synthesis of oligonucleotide or PNA arrays to achieve accurate detection and quantification of cancer mutations. Several groups have attempted to make oligonucleotide arrays with various degrees of success [33, 37- 40]. These approaches may be divided into three categories: (i) Synthesis of oligonucleotides by standard methods and their attachment one at a time in a spatial array [38-40] (ii) Photolithographic masking and photochemical deprotection on a silicon chip, to allow for synthesis of short oligonucleotides [37], and (iii) Physical masking to allow for synthesis of short oligonucleotides by addition of single bases at the unmasked areas [33, 36]. Although considerable progress has been made in constructing oligonucleotide arrays, some containing as many as 256 independent addresses, severe limitations have been noted in using these arrays for detecting specific DNA sequences by hybridizations. Arrays containing longer oligonucleotides can currently be synthesized only by attaching one address at a time, and thus are limited in potential size. (Current methods for attaching an oligonucleotide take about 1 hour, thus an array of 1,000 addresses would require over 40 days of around-the-clock work to prepare.) The "reverse dot blot" approach is capable of distinguishing single base differences in homozygous or heterozygous individuals, as well as the presence of a *ras* mutation diluted 20-fold by normal DNA [40]. However, hybridization methods require careful attention to temperature and salt conditions, and cannot achieve the high sensitivity of the cancer detection methods described in this proposal. Arrays containing large numbers of short oligonucleotides have performed significantly better on the computer than in practice. Syntheses on membranes or silicon chips are plagued by less than 100% efficiency, effectively limiting the size of these oligonucleotides to 8- to 10-mers. Imperfect hybridizations generate significant background signals, which severely hamper use of these arrays for DNA sequencing [36].

(b) *Zip code concept.* This proposal introduces a novel approach to oligonucleotide arrays which should obviate the above problems. One significant difference between our approach and literature array methods is that we use the array as a means to capture the *correctly generated* product. While others try to distinguish closely related sequences by subtle differences in melting temperatures during hybridization, we have already achieved the required exquisite specificity due to the discriminating actions of thermostable ligase in solution. Thus, our arrays can be designed to contain sequences which are *very different* from each other. Our array may be likened to 1,000 different antibodies that bind 1,000 different antigens with tight binding constants and no cross-reactivity. These arrays are completely universal, so that a single design may be used in detection of infectious and genetic diseases, or cancers. Best of all, the arrays will be highly stable and reusable.

The 1,000 different "antigens" are unique 24-mer "zip code" sequences linked covalently to the approximately 20- to 25-mer target-specific sequence of an LDR primer. A "zip code" sequence does not have any homology to either the target sequence or to other sequences on the genome. This zip code tail is then captured by its "antibody", a sequence complementary to the zip code on the addressable solid support array. The concept is shown in two possible formats for detection of the p53 R248 mutation (Fig. 2). At the top of the diagram shows two alternative formats for primer design to identify the presence of a germ line mutation in codon 248 of the p53 tumor suppression gene. The wild type sequence codes for arginine (R248) while the cancer mutation codes for tryptophan (R248W). The lower part of the diagram is a schematic for zip code capture. In the first format (A), the discriminating primers contain the allelic specific base (T for mutant and C for wild type) on the 3' end and 24-mer zip codes Z1 and Z2 on their 5' ends respectively. A common downstream primer contains a fluorescent group F1 at its 3' end. In the presence

presence of appropriate target DNA (wild type DNA is shown), the correct ligation products form. After hybridization of the zip code primers to their complementary sequences on the addressable array, unreacted fluorescent primers will be washed away. Mutant and wild type signal may be quantified using a FluorImager, and distinguished by their position on the array. In an alternative format (B), the discriminating oligonucleotides contain two different fluorescent groups F1 and F2, while the common oligonucleotide contains the zip code Z1. In this format, mutant and wild type signal are distinguished by the differences in fluorescence between F1 and F2 (see legend of Fig. 2 for more details).



**Fig. 2.** Two alternative formats for zip code capture (see following page for legend).

Two alternative formats for zip code capture (legend for previous page). The top portion of the diagram shows two alternative formats for primer design to identify the presence of a germ line mutation in codon 248 of the p53 tumor suppressor gene. The wild type sequence codes for arginine (R248), while the cancer mutation codes for tryptophan (R248W). The bottom part of the diagram is a schematic diagram of zip code capture. The thick horizontal line depicts the membrane or solid surface containing the addressable array. The thin curved lines indicate a flexible linker arm. The thicker lines indicate a PNA sequence, attached to the solid surface in the C to N direction. For illustrative purposes, the PNA oligonucleotides are drawn vertically, making the linker arm in section B appear "stretched". Since the arm is flexible, the oligonucleotide will be able to hybridize 5' to C and 3' to N in each case, as dictated by base pair complementarity. A similar orientation of DNA/PNA hybridization would be allowed if the PNA were attached to the membrane at the N-terminus. Similar considerations apply when the complementary zip code on the support is a DNA oligonucleotide rather than PNA. (A) Two LDR primers are designed to discriminate wild type and mutant p53 by containing the discriminating base C or T at the 3' end. In the presence of the correct target DNA and *Tth* ligase, the discriminating primer is covalently attached to a common downstream oligonucleotide. The downstream oligonucleotide is fluorescently labeled. The discriminating oligonucleotides are distinguished by the presence of a unique "zip code" sequences, Z1 and Z2, at each of their 5' ends. A black dot indicates that target dependent ligation has taken place. After ligation, all zip code primers may be captured by their complementary "zip code" sequences at unique addresses on the array. Both ligated and unreacted primers are captured by the PNA array. Unreacted fluorescently labeled common primers and target DNA are then washed away at a high temperature (approximately 65°C to 80°C) and low salt. Mutant signal is distinguished by detection of fluorescent signal at the Z1 position, while wild type signal appears at the Z2 position. Heterozygosity is indicated by equal signals at both Z1 and Z2. The signals may be quantified using a Molecular Dynamics FluorImager. This format uses a unique address for each allele, and may be preferred for achieving very accurate detection of low levels of signal (30 to 100 attomoles of LDR product). (B) In this format, the discriminating oligonucleotides are distinguished by having different fluorescent groups, F1 and F2, on their 5' end. Either oligonucleotide may be ligated to a common downstream oligonucleotide containing a zip code sequence Z1 on its 3' end. In this format, both wild type and mutant LDR products are captured at the same address on the array, and are distinguished by their different fluorescence. This format allows for a more efficient use of the array and may be preferred when trying to detect hundreds of potential germline mutations.

(c) *Design and synthesis of arrays.* This Research Plan will explore variations of two general approaches for synthesizing arrays. In the first approach, we will prepare full-length 24-mer DNA oligonucleotides or PNA oligomers, which are subsequently linked covalently to a solid support or membrane. Alternatively, the deprotected DNA or PNA may remain linked to the bead, and the entire bead glued to a solid support. In the second approach, 36 specially designed PNA tetramers will be synthesized. These tetramers will be added to specific rows or columns on a solid support or membrane surface. The resulting "checkerboard" pattern will generate unique addressable arrays of PNA 24-mers.

We will initially explore glass and derivatized membrane supports to test their sensitivities and capacities as array surfaces. Pilot experiments will involve synthesis of five zip code PNA oligomers or oligonucleotides (sequences listed in Table 2, later). These oligomers will be covalently linked to the test surfaces. Fluorescently labeled complementary DNA zip code sequences will be synthesized in Core B, and used for testing arrays produced in this project. Note that for the initial studies, we do not require the longer conjugates that combine the LDR primer with the zip code (see Fig. 1).

What properties are desired in an array? The most important factor is good loading of oligonucleotide or PNA oligomer in a relatively small, but well-defined area. The current commercially available fluorescent imager can detect a signal as low as 2 attomoles per 50μ square pixel. Thus, a reasonable size address or "spot" on an array would be about 4 x 4 pixels, or 200μ square. The limit of detection for such an address would be about 32 attomoles per "spot", which is comparable to the 100 attomole detection limit using a DNA sequencing machine. The capacity of oligonucleotide which can be loaded per 200μ square will give an indication of the potential signal to noise ratio. A loading of 20 fmoles would give a signal to noise ratio of 625 to 1, while 200 fmoles would allow for a superb signal to noise ratio of 6,250 to 1. Loadings in excess of 200 fmoles will be unnecessary, since most LDR reactions use only 200 fmoles of each primer. The oligonucleotide or PNA oligomer should be on a flexible "linker arm" and on the "outside" or "surface" of the solid support for easier hybridizations. The support should be non-fluorescent, and should not interfere with hybridization nor give a high background signal due to nonspecific binding. In a mode where bead(s) are attached (typical size 50-200μ), neither beads nor "glue" should give a high background signal due to nonspecific binding or intrinsic fluorescence.

This proposal also introduces a novel approach for the design and synthesis of a universal PNA oligonucleotide array with 1,296 addresses. We envision each address to be about 200μ with an equal size



space in between addresses. Feasibility will be assessed with a 25 address array that is about 2 mm square; the full-sized array would be about 1.4 cm square. Preparation of such arrays (pilot and full-sized) will be carried out as a joint project with our industrial collaborators Dr. James Coull and his team at Millipore, and Dr. Ronald Cook who heads Siris Labs. (Please see letters of collaboration in overview section). The required preliminary synthesis in the academic laboratories will use a Biorad dot blot apparatus which contains individual microtiter wells sandwiched around a membrane. This allows for addition and filtration of chemicals in each well.

## (ii) Design and optimization of zip codes and addresses

(a) *General considerations.* The principle of using zip codes has been explained earlier. The complementary zip codes (addresses) on the solid supports can be either DNA or PNA. *Both* will be tested. However, we expect that PNA-based capture of zip codes may have advantages over DNA-based capture because PNA/DNA duplexes are much stronger than DNA/DNA duplexes, by about 1°C/base-pair [44]. Thus, for a 24-mer DNA/DNA duplex with  $T_m = 72^\circ\text{C}$ , the corresponding duplex with one PNA strand would have a "predicted"  $T_m = 96^\circ\text{C}$  (the actual melting point might be slightly lower as the above "rule of thumb" is less accurate as melting points get over  $80^\circ\text{C}$ ). Additionally, the melting difference between DNA/DNA and PNA/DNA becomes even more striking at low salt.

(b) *Enhancement of the hybridization affinity of zip code/address duplexes.* The melting temperature of DNA/DNA duplexes can be estimated as  $[4n(\text{G}\cdot\text{C}) + 2m(\text{A}\cdot\text{T})]^\circ\text{C}$ . If possible, we would like to narrow the  $T_m$  difference between zip code duplexes resulting from differences in G•C/A•T content, and in this way further optimize zip code capture. Froehler has shown that use of 5-propynyl-dU in place of thymine increases the  $T_m$  of DNA duplexes an average of  $1.7^\circ\text{C}$  per substitution [51]. We suggest that the same substitution in the zip code capture scheme would lower the  $T_m$  difference between zip code/address duplexes, and raise the  $T_m$  for all of the zip code/address duplexes. Phosphoramidite derivatives of 5-propynyl-dU (Fig. 3) will be prepared according to Froehler [51]. The 5-propynyluracil PNA monomer with Fmoc amino protection will be made (Fig. 4) following the published synthesis of PNA monomers [41, 42], replacing thymine with 5-iodouracil and using Pd(0) coupling of the alkylated 5-iodouracil and propyne. These monomers will be incorporated into synthetic DNA and PNA strands, respectively, and evaluated as described later.

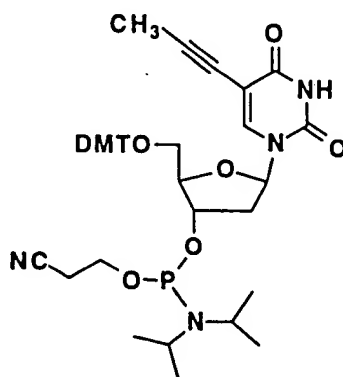
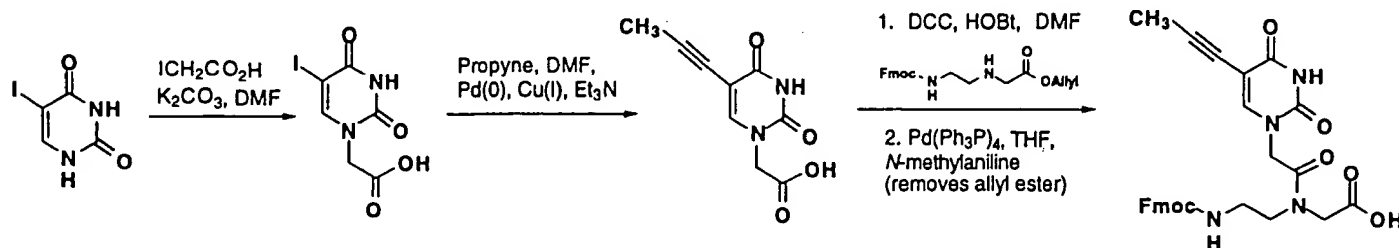


Fig. 3. Structure of nucleoside analogue 5-propynyl-dU.





The Boc-protected derivative could be made by a similar route.

**Fig. 4.** Synthesis of Fmoc-protected 5-propynyl-uridine PNA monomer.

(c) *Zip code sequences designed from tetramer building blocks.* Of the 256 ( $4^4$ ) possible ways in which four bases can be arranged as tetramers, we have selected 36 that have unique sequences (Fig. 5). Each of the chosen tetramers differs from all the others by at least two bases, and no two dimers are complementary to each other. Furthermore, tetramers that would result in self-pairing or hairpin formation of the addresses have been eliminated (see legend to Fig. 5 for further details of the design process).

The final tetramers are listed in Table 1, and have been numbered arbitrarily from 1 to 36. Our premise is to use this unique set of tetramers as design modules for the required 24-mer zip code and 24-mer address sequences. The structures can be assembled by stepwise (one base at a time) or convergent (tetramer building blocks) synthetic strategies. Note that the numbering scheme for tetramers allows us to abbreviate each zip code as a string of six numbers (e.g., second column of Table 2, in following section).

(d) *Initial zip code test sequences.* The concept of zip code 24-mers designed from a unique set of 36 tetramers (Table 1) allows a huge number of possible structures,  $36^6 = 2,176,782,336$ . We have chosen five structures (Table 2) that have nearly equal G + C content as targets for the graded set of studies that are needed to establish the proposed methodology.

(e) *Solution studies of zip code annealing.* Our eventual goal is to exploit zip code hybridization to direct fluorescently labelled LDR products towards specific addresses on a solid support. However, first we wish to validate duplex formation in solution. Test sequences (Table 2) will be used. The  $T_m$  of each duplex will be measured by recording the  $A_{260}$  of the oligonucleotide solutions ( $\sim 5\mu\text{M}$  concentration of each single strand) versus temperature.

Synthetic probes (normal and complementary directions) for the aforementioned studies will be prepared as either DNA or PNA, with either all thymine or all 5-propynyl-uracil. Where syntheses are straightforward, they will be performed by Core B, but where methodology is still under development, syntheses will be performed in the laboratories of program project chemists or industrial collaborators. These syntheses will generate for each sequence a total of eight oligomers, which can be combined in 16 ways that form duplexes.

2nd two bases

1st two bases

	TT	TC	TG	TA	CT	CC	CG	CA	GT	GC	GG	GA	AT	AC	AG	AA
TT							16'			23'		TTGA 6			TTAG 8	
TC			TCTG 1		30'	TCCC 3			TCGT 5							6'
TG		TGTC 2		36'			TGCG 4						TGAT 7		11'	
TA						18'		TACA 36			33'					
CT	32'		CTTG 9					CTCA 11	CTGT 13							8'
CC				CCTA 33					29'				CCAT 15			
CG	CGTT 10		12'					4'					28'			CGAA 16
CA		34'			25'		CACG 12			CAGC 14		1'			9'	
GT					GTCT 19	24'							31'			
GC	GCTT 17		14'											22'		GCAA 23
GG		20'		GGTA 18	35'							3'		GGAC 24		
GA			GATG 34			GACC 20		2'	GAGT 21							
AT							ATCG 28	7'			15'			ATAC 31		
AC		21'			ACCT 27						ACGG 29	5'			13'	
AG			AGTG 25			AGCC 35			27'			AGGA 30		19'		
AA		AATC 26					10'			17'					AAAG 32	

**Fig. 5.** Design of 36 tetramers which differ from each other by at least 2 bases. Checkerboard pattern shows all 256 possible tetramers. A given square represents the first two bases on the left followed by the two bases on the top of the checkerboard. Each tetramer must differ from each other by at least two bases, and should be non-complementary. The tetramers are shown in the white boxes, while their complements are listed as (number)'. Thus, the complementary sequences GACC (20) and GGTC (20') are mutually exclusive in this scheme. In addition, tetramers must be non-palindromic, e.g., TCGA (darker diagonal line boxes), and non-repetitive, e.g., CACA (darker diagonal line boxes from upper left to lower right). All other sequences which differ from the 36 tetramers by only 1 base are shaded in light gray. Four potential tetramers were not chosen as they are either all A•T or G•C bases. In addition, thymine can be replaced by 5-propynyl uridine when used within DNA or PNA address sequences as well as in the DNA zip code sequences. This would increase the  $T_m$  of an A•T base pair by  $\sim 1.7^\circ\text{C}$ . Thus,  $T_m$  values of individual tetramers should be approximately  $15.1^\circ\text{C}$  to  $15.7^\circ\text{C}$ .  $T_m$  values for the full length 24-mers should be  $95^\circ\text{C}$  or higher.

**Table 1.** List of tetramer PNA sequences and complementary DNA sequences, which differ from each other by at least 2 bases.

Number	Sequence (N-C)	Complement (5'-3')	G + C
1.	TCTG	CAGA	2
2.	TGTC	GACA	2
3.	TCCC	GGGA	3
4.	TGCG	CGCA	3
5.	TCGT	ACGA	2
6.	TTGA	TCAA	1
7.	TGAT	ATCA	1
8.	TTAG	CTAA	1
9.	CTTG	CAAG	2
10.	CGTT	AACG	2
11.	CTCA	TGAG	2
12.	CACG	CGTG	3
13.	CTGT	ACAG	2
14.	CAGC	GCTG	3
15.	CCAT	ATGG	2
16.	CGAA	TTCG	2
17.	GCTT	AAGC	2
18.	GGTA	TACC	2
19.	GTCT	AGAC	2
20.	GACC	GGTC	3
21.	GAGT	ACTC	2
22.	GTGC	GCAC	3
23.	GCAA	TTGC	2
24.	GGAC	GTCC	3
25.	AGTG	CACT	2
26.	AATC	GATT	1
27.	ACCT	AGGT	2
28.	ATCG	CGAT	2
29.	ACGG	CCGT	3
30.	AGGA	TCCT	2
31.	ATAC	GTAT	1
32.	AAAG	CTTT	1
33.	CCTA	TAGG	2
34.	GATG	CATC	2
35.	AGCC	GGCT	3
36.	TACA	TGTA	1

**(iii) Solid support materials for array technology**

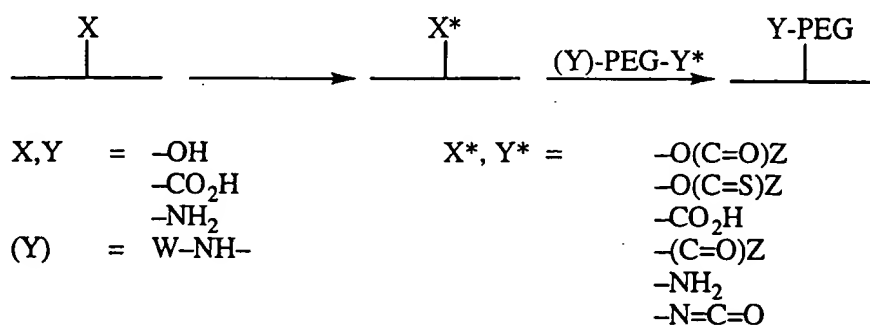
Earlier sections of this proposal have reviewed the ideal requirements for array support materials, in the context of options from the literature and our own extensive experiences. The solid supports must be charged with DNA oligonucleotides or PNA oligomers; this is achieved either by attachment of pre-synthesized probes, or by direct assembly and side-chain deprotection (without release of the oligomer) onto the support. Further, the support environment needs to be such as to allow efficient hybridization. Towards this end, three factors may be identified: (i) sufficient hydrophilic character of support material (e.g., PEG or carbohydrate moieties); (ii) flexible linker arms (e.g., hexaethylene oxide or longer PEG chains) separating the probe from the support backbone; (iii) "shaving" procedures which allow probe immobilization or probe synthesis to occur only in the most accessible "surface" areas of the support. It should be kept in mind that numerous ostensibly "flat surfaces" are quite thick at the molecular level. Lastly, it is important that the support material not provide significant background signal due to non-specific binding or intrinsic fluorescence.

**Table 2.** List of initial PNA zip code and complementary DNA oligonucleotides.

Polymer	Zip code	Sequence	G+C
PNA	16-3-34-2-9-1	NH <sub>2</sub> - <sup>16</sup> CGAA- <sup>3</sup> TCCC- <sup>34</sup> GATG- <sup>2</sup> TGTC- <sup>9</sup> CTTG- <sup>1</sup> TCTG-COOH	13
DNA	1-9-2-34-3-16(c)	5'-CAGA-CAAG-GACA-CATC-GGGA-TTCG-3'	13
PNA	7-3-11-2-18-1	NH <sub>2</sub> - <sup>7</sup> TGAT- <sup>3</sup> TCCC- <sup>11</sup> CTCA- <sup>2</sup> TGTC- <sup>18</sup> GGTA- <sup>1</sup> TCTG-COOH	12
DNA	1-18-2-11-3-7(c)	5'-CAGA-TACC-GACA-TGAG-GGGA-ATCA-3'	12
PNA	20-3-14-2-7-1	NH <sub>2</sub> - <sup>20</sup> GACC- <sup>3</sup> TCCC- <sup>14</sup> CAGC- <sup>2</sup> TGTC- <sup>7</sup> TGAT- <sup>1</sup> TCTG-COOH	14
DNA	1-7-2-14-3-20(c)	5'-CAGA-ATCA-GACA-GCTG-GGGA-GGTC-3'	14
PNA	29-3-23-2-12-1	NH <sub>2</sub> - <sup>29</sup> ACGG- <sup>3</sup> TCCC- <sup>23</sup> GCAA- <sup>2</sup> TGTC- <sup>12</sup> CACG- <sup>1</sup> TCTG-COOH	15
DNA	1-12-2-23-3-29(c)	5'-CAGA-CGTG-GACA-TTGC-GGGA-CCGT-3'	15
PNA	13-35-27-33-2-7	NH <sub>2</sub> - <sup>13</sup> CTGT- <sup>35</sup> AGCC- <sup>27</sup> ACCT- <sup>33</sup> CCTA- <sup>2</sup> TGTC- <sup>7</sup> TGAT-COOH	12
DNA	7-2-33-27-35-13(c)	5'-ATCA-GACA-TAGG-AGGT-GGCT-ACAG-3'	12

A variety of materials, which include suitably modified glass, plastic, or cellulose surfaces, PEG-PS beads, or a variety of membranes, will be examined in the context of the needs summarized above. These materials will be obtained from commercial sources or from our industrial collaborators (Dr. James Coull at Millipore, Dr. Ronald Cook at Siris), or else will be prepared in our laboratories by following literature precedents. Depending on the material, surface functional groups (i.e., hydroxyl, carboxyl, amino) may be present from the outset (perhaps as part of the coating polymer), or will require a separate procedure (e.g., plasma amination, chromic acid oxidation, treatment with a side-chain functionalized alkyltrichlorosilane) for introduction of the functional group. Hydroxyl groups become incorporated into stable carbamate (urethane) linkages by several methods. Amino functions can be acylated directly, whereas carboxyl groups are activated, e.g., with N,N'-carbonyldiimidazole or water-soluble carbodiimides, and reacted with an amino-functionalized compound (Fig. 6). Unreacted amino groups will be blocked by acetylation or succinylation, to ensure a neutral or negatively charged environment that "repels" excess unhybridized DNA. Loading levels will be determined by standard analytical methods [47].

Often, it will be desirable to introduce a PEG spacer with complementary functionalization, prior to attachment of the starting linker for DNA or PNA synthesis. The methodology to do so is in hand [22, 23, 52] and will be pursued alongside with control experiments on the same materials lacking PEG. Similarly, dextran layers can be introduced as needed by precedented chemistries [27, 53]. Finally, enzymatic "shaving" is carried out readily by our recently developed procedure using chymotrypsin to cleave a short substrate that is distributed uniformly throughout a bead or on a derivatized surface. In our studies on peptide/receptor (antibody or binding protein) interactions, we have shown that shaving protocols expose a relatively small portion (approximately 1 to 5%) of the total functional groups, yet they reach all receptor-accessible sites [46]. We plan to establish whether the same site selectivity can be achieved for hybridization reactions, and compare the results to controls run on "unshaven" materials.



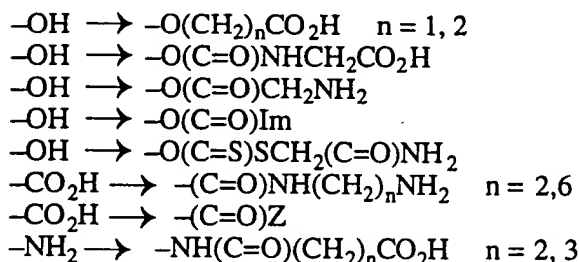
W = protecting group, e.g. Boc, Fmoc

Z = activating group, e.g. imidazole (Im), *p*-nitrophenol (OPnp),  
hydroxysuccinimide (OSu), pentafluorophenol (OPfp)

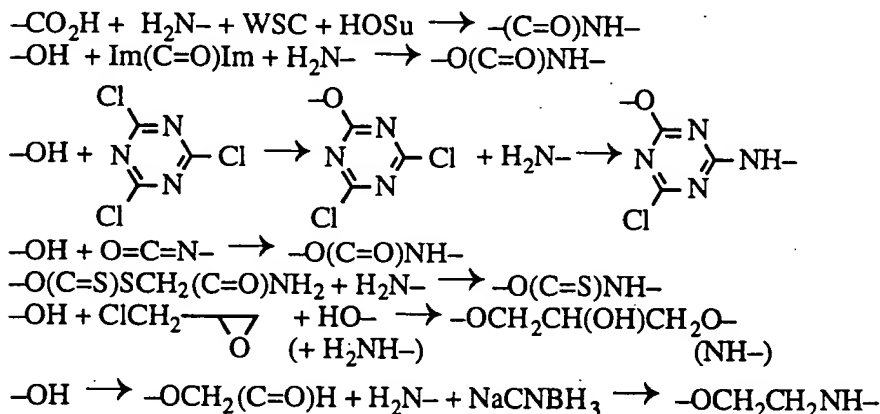
PEG = oligo or poly(ethylene glycol), backbone  $(\text{CH}_2\text{CH}_2\text{O})_n$   $n = 6$  to  $200$   
(can also be grown by anionic polymerization with  $\nabla$ )

WSC = water soluble carbodiimide

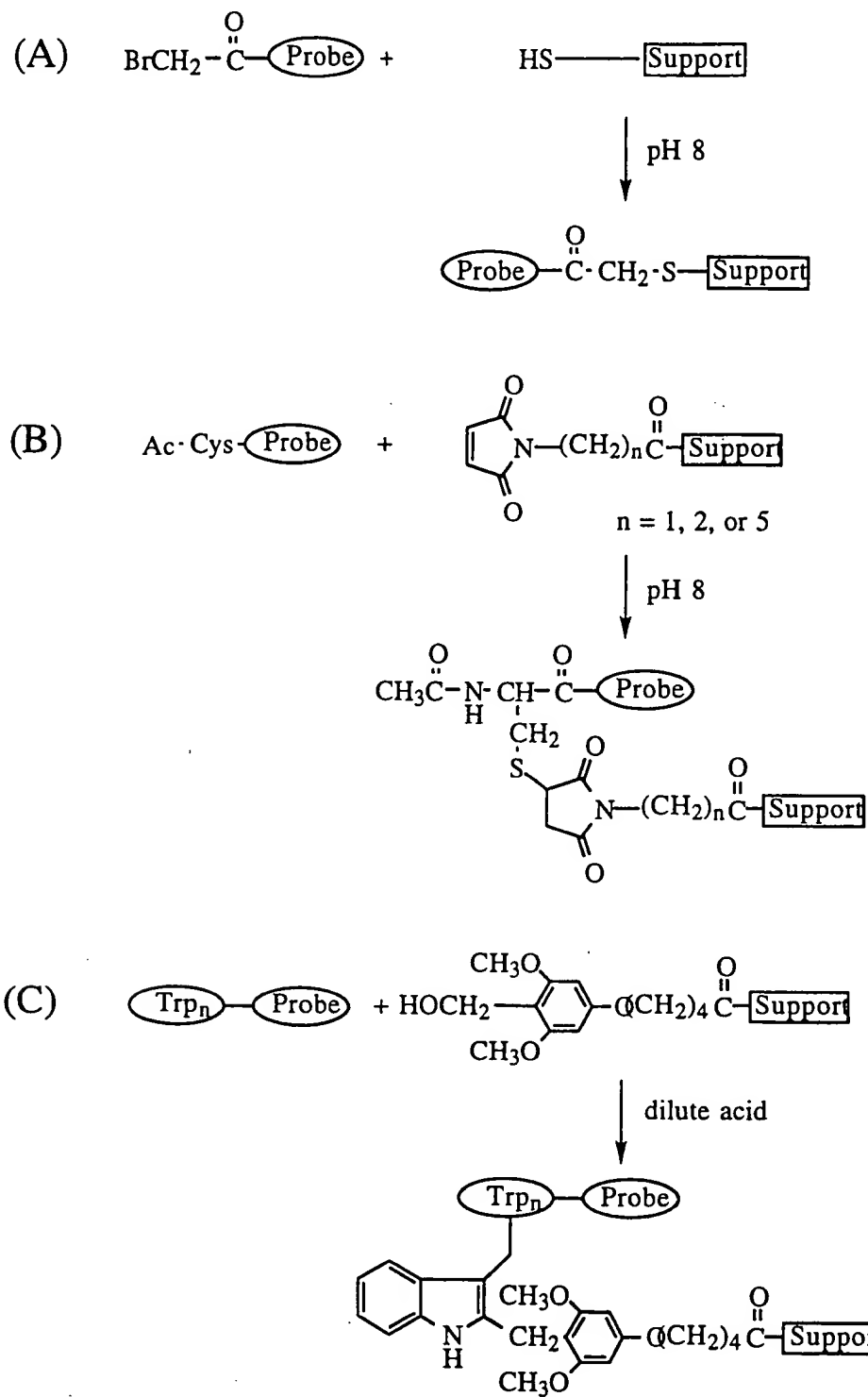
**Functional group transformations/activation (as needed),  $\text{X} \rightarrow \text{X}^*$ ,  $\text{Y} \rightarrow \text{Y}^*$**



**Covalent linkage,  $\text{X}^* + \text{Y}^*$**



**Fig. 6.** Chemical reactions for covalent modifications, grafting, and oligomer attachments to solid supports. The solid supports can be beads, membranes, or surfaces, with a starting functional group X. Functional group transformations can be carried out in a variety of ways (as needed) to provide group X\* which represents one partner in the covalent linkage with group Y\*. The Figure shows specifically the grafting of PEG, but the same repertoire of reactions can be used (however needed) to attach carbohydrates (with hydroxyl), linkers (with carboxyl), and/or DNA oligonucleotides and PNA oligomers that have been extended by suitable functional groups (amino or carboxyl). In some cases, group X\* or Y\* is pre-activated (isolable species from a separate reaction); alternatively, activation occurs in situ. Referring to PEG as drawn in the Figure, Y and Y\* can be the same (homobifunctional) or different (heterobifunctional); in the latter case, (Y) can be protected for further control of the chemistry.



**Fig. 7.** Proposed chemistries for covalent attachment of DNA or PNA probes to solid supports. Chemically synthesized probes can be extended, on either end (shown here on the N-terminal of PNA or the 5' end of DNA). Further variations of the proposed chemistries are readily envisaged. (A) An amino group on the probe is modified by bromoacetic anhydride; the bromoacetyl function is captured by a thiol group on the support. (B) An N-acetyl, S-tritylcysteine residue coupled to the end of the probe provides, after cleavage and deprotection, a free thiol which is captured by a maleimido group on the support. (C) The probe contains an oligo-tryptophanyl tail ( $n = 1$  to 3), which is captured after treatment of a HAL-modified support with dilute acid.

**(iv) Immobilization of individually synthesized DNA oligonucleotides or PNA oligomers onto solid supports**

(a) *Synthesis of 24-mer DNA oligonucleotides and 24-mer PNA oligomers.* The five sequences listed in Table 2 are designed for testing the hybridization properties of zip code arrays. They will be synthesized by Core B as DNA oligonucleotides using standard phosphoramidite chemistries [29], and incorporating an "aminolink" group at the 5'-terminus. In addition, the same sequences will be synthesized as PNA oligomers by stepwise Boc or Fmoc solid-phase chemistry, or by a segment condensation approach using suitably protected PNA tetramers. The PNA will have one endgroup blocked (e.g., acetyl on N-terminus, or amide on C-terminus), and the other terminus extended with  $\epsilon$ -aminocaproic acid to provide a free aliphatic amino or carboxyl site for ultimate linking to the solid support. The complementary zip code oligonucleotide sequences will be prepared with a fluorescent "Fam" group at the 5'-end, by Core B.

Synthetic DNA oligonucleotides or PNA oligomers will be released from the resin supports, concurrent with removal of side-chain protecting groups. These modified oligomers will be purified to homogeneity by well-precedented polyacrylamide gel electrophoresis (PAGE) or high performance liquid chromatography (HPLC: reversed-phase or anion-exchange) procedures. Immobilization to solid supports will follow, as described below.

(b) *Covalent attachment of DNA oligonucleotides or PNA oligomers to solid supports.* The purified oligomers all contain a free aliphatic amino group at the terminus, which allows attachment to a derivatized membrane according to Zhang [40]. Other attachment chemistries based on amino group chemistry will also be pursued, building on a wealth of precedents for connecting functionalized polymers and proteins to each other and to solid matrices [54] (Fig. 6). These procedure can be carried out in series with several probes, resulting in site-specific attachments. Once the complementary zip code probes have been immobilized, oligonucleotide hybridizations using fluorescently labeled zip codes will be carried out to evaluate both capacity and signal to noise ratio, as described in Core B.

Encouraging results in the experiments outlined above would provide impetus to the exploration of additional immobilization ("capture") chemistries, which need to be rapid, specific, and non-destructive to the combination of functional groups found in DNA oligonucleotides and PNA oligomers. Our strategy involves incorporation, through synthesis, of alternative functional groups at either end of the probe, together with modification of the support by a suitable complementary functional group. More specifically, we can take advantage of the facile S-alkylation or Michael addition of thiol groups, or of the reaction in dilute acid of indole moieties with tris(alkoxy)benzyl carbocations (Fig. 7).

**(v) Synthesis of oligonucleotides or PNA oligomers on solid supports and creation of arrays on solid surfaces**

(a) *Synthesis of 24-mer DNA oligonucleotides and 24-mer PNA oligomers on "shaved" beads.* A second approach to constructing the arrays required for zip code capture starts with the assumption that suitable probes can be assembled and side-chain deprotected with covalent retention on beads used for solid-phase synthesis; these beads are then delivered to discrete addresses on a solid surface. General considerations have been outlined earlier; we consider this mode to represent a particularly pertinent case where application of our "shaving" concept may be critical to success. As before, the five sequences listed in Table 2 will be synthesized, either as DNA oligonucleotides (standard phosphoramidite chemistry) or as PNA oligomers (stepwise Boc or Fmoc chemistry).

PEG-PS beads of 100 $\mu$  diameter have a normal capacity of approximately 30 pmol, meaning that a shaved bead is predicted to hold about 0.1 to 0.5 pmol of final product. This level of material is well within the requirements of the subsequent hybridization studies. Chymotryptic "shaving" of a Boc-Trp-Gly-PEG-PS sequence generates a free  $\alpha$ -amino group from "surface" glycine residues. For PNA synthesis, the C-terminal monomer is coupled to form a non-cleavable peptide bond; for DNA synthesis, an N-acetyl-serine spacer is introduced so that phosphoramidite synthesis begins off the free hydroxyl side-chain. For DNA chains upon completion of chain assembly, removal of the usual base-labile side-chain and phosphate protecting groups with aqueous ammonia yields the free probe oligomer linked covalently to the outside

areas of PEG-PS. For PNA oligomers, benzyloxycarbonyl-type protecting groups will be removed with strong acid, e.g., trifluoromethanesulfonic acid (the strategy may change if/when milder protection schemes under development at Millipore or by us are established).

In order to better document the chemistry of chain assembly on "shaved" as well as control beads, the synthesis outlined above can be modified by introduction of base-stable, orthogonally cleavable linkers to separate the free glycine from the 3' or C-terminal residue. Suitable choices (Fig. 8) include acid-labile *p*-alkoxybenzyl (PAB), photolabile *o*-nitrobenzyl (ONb), or Pd(0)-cleavable allyl (Al) [47, 48]. The oligonucleotide or PNA products can be released from the support in a discrete step, and evaluated by standard analytical criteria as well as solution hybridization with the complementary sequences.

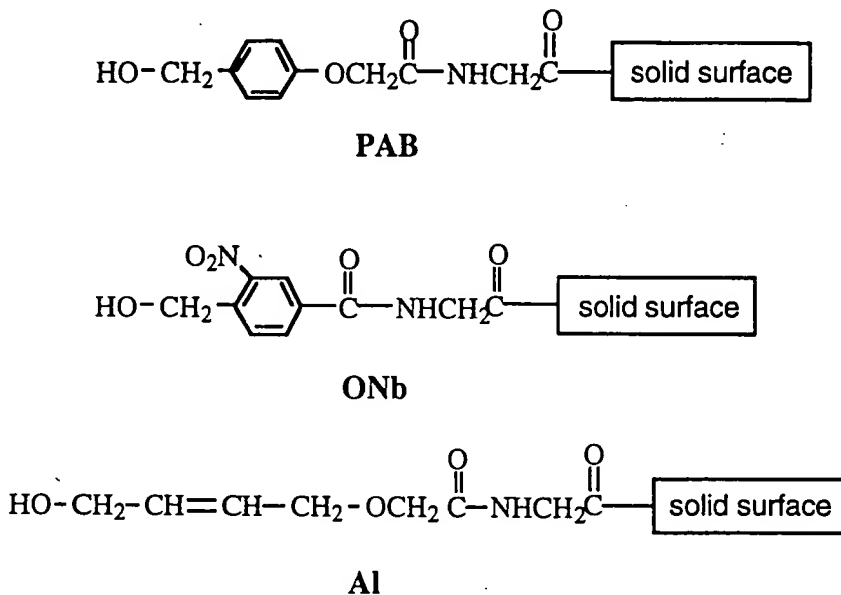


Fig 8. Handles for attachment of oligopolymers to "shaved" beads. Handles are coupled through their carboxyl groups to "shaved" beads. The free hydroxyl on the left side of each structure can be esterified with the C-terminal PNA monomer or phosphitylated with a nucleoside phosphoramidite.

(b) *Attachment of beads to solid surfaces.* DNA or PNA synthesis on PEG-PS using automated instrumentation is generally carried out with several hundred thousand to several million beads, setting the stage for economical mass production of arrays. Each 200 $\mu$  square pixel will contain several 100 $\mu$  beads, and different spatial addresses will contain different beads. Several of our industrial collaborators are actively pursuing concepts for attaching beads to solid surfaces in a rapid and automated manner. (Please see letters of collaboration from Dr. James Coull of Millipore and Dr. Ronald Cook of Siris Labs). Briefly, these concepts include precise positioning of the bead over a gridded surface and melting one surface to attach them, using grooves or dimples in the surface to help position beads (using vacuum suction to guide a bead into a particular position), and/or projecting the beads onto a surface containing glue. For academic purposes, we will place beads onto a thin layer of some bonding material, such as epoxy. All bonding materials will need to be tested for resistance to high temperatures/high salt conditions, and to confirm the absence of non-specific binding to DNA oligonucleotides. Oligonucleotide hybridizations using fluorescently labeled complementary zip codes will be used to evaluate both capacity and signal to noise ratio as described in Core B.

(c) *Variations.* As necessary, the evolving literature methods for simultaneous syntheses of peptides or oligonucleotides at defined positions will be adapted in concert with the chemistry (linkers, protection strategies) and other concepts (shaving) presented above for beads. For example, it will be of interest to learn whether shaving of membranes, PEG-modified polyethylene surfaces, or pins helps with synthesis and/or hybridization efficiency. The various literature protocols for multiple synthesis are quite labor-intensive, but they may need to be pursued should we be unable to devise successful way to glue beads that contain DNA or PNA probes to surfaces, and to apply such materials for hybridization.



**(vi) Direct synthesis of PNA arrays by masking/segment condensation on solid supports**

(a) *Perspective.* In principle, arrays can be constructed most effectively by use of highly accurate masking and unmasking technology with per cycle yields of close to 100%. Unfortunately, current chemistries proceed in at best 97% yield per step, with a possible further drop-off as chain length increases. These relatively low efficiencies allow for construction of modest arrays in the octamer to decamer size range (even so, with substantial synthetic error rates which translate to hybridization at false addresses), but preclude construction of 24-mers needed as complementary zip codes in the cancer detection scheme of this program project proposal.

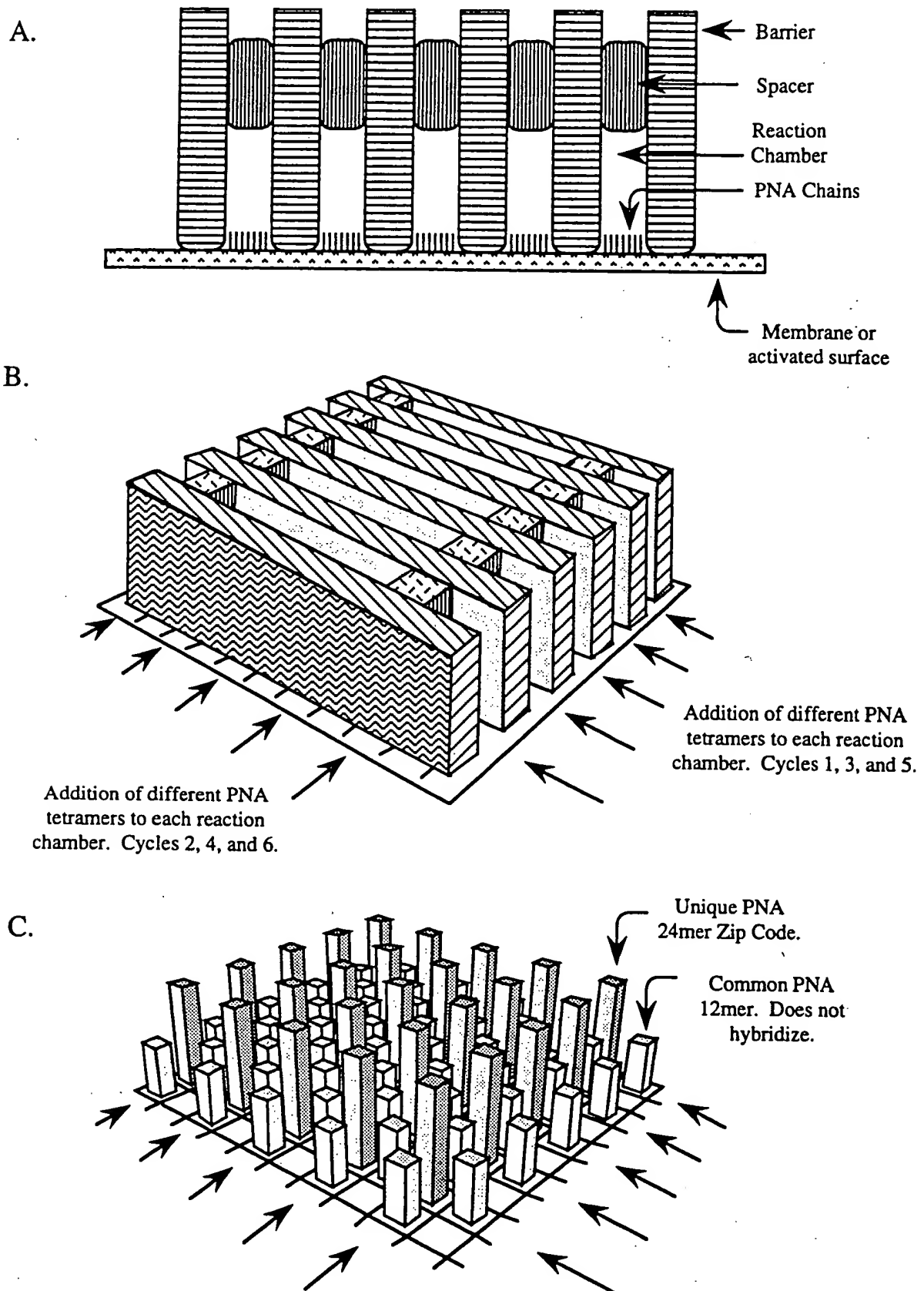
In the following, we propose a novel way to circumvent the aforementioned problems. The design of zip code arrays (in which individual arrays have substantial differences to minimize any chances of cross-reactivity; see earlier Fig. 5 and Table 1, and accompanying discussion) has been *integrated* with the synthetic strategy. Rather than carrying out stepwise synthesis to introduce bases one at a time, we use protected PNA tetramers as building blocks. (As shown below, these are easy to prepare; the corresponding protected oligonucleotide intermediates would require additional protection of the internucleotide phosphate linkages.) Construction of the 24-mer at any given address requires only six synthetic steps, with a likely improvement in overall yield by comparison to stepwise synthesis. Moreover, since failure sequences at each address are shorter and lacking at least four bases, there is no risk that these will interfere with correct hybridization or lead to incorrect hybridizations. This insight also means that "capping" steps will not be necessary.

Masking technology will allow several addresses to be built up simultaneously, as is explained below. As direct consequences of the manufacturing process for the arrays, several further advantages are noted. Each 24-mer address differs from its nearest 24-mer neighbor by three tetramers, or at least 6 bases. At low salt, each base mismatch in PNA/DNA hybrids decreases the melting temperature by 8°C. Thus, the  $T_m$  for the correct PNA/DNA hybridization is at least 48°C higher than any incorrect hybridization. Also, neighboring 24-mers are separated by 12-mers, which do not hybridize with anything and represent "dead" zones in the cancer detection profile. Finally, by choosing PNA addresses, we create rugged, reusable arrays.

The remaining description indicates methods for preparation of 36 unique PNA tetramers, and shows the mechanical/chemical strategy to prepare the arrays. Pilot experiments will result in the creation of a 5x5 array with 25 addresses of PNA 24-mers. Ultimately, all 36 tetramers can be incorporated to generate full-size arrays of 1,296 addresses.

(b) *Synthesis of protected PNA tetramer building blocks.* For each of the 36 unique sequences that have been designed (Table 1), we require the intermediates with appropriate protection on the  $\alpha$ -amino group, on the side-chains, and with a free  $\alpha$ -carboxyl group. This can be done readily by Fmoc chemistry on PAB or HAL resins, according to standard protocols published from our laboratory [50, 55]. Following cleavage in dilute acid, the protected intermediates will be purified by chromatography. Alternatively, we can use Boc chemistry with ONb (photolabile) or allyl (cleaved by Pd(0)) resins [47, 48, 56].

(c) *Construction of PNA arrays.* As stated already, only the pilot study with a 5x5 array is described. Considerations concerning the solid support are the same as described earlier: starting surfaces will contain free amino groups ("shaved" if necessary), a non-cleavable amide linkage will connect the C-terminus of PNA to the support, and orthogonal side-chain deprotection must be carried out upon completion of segment condensation assembly in a way that PNA chains are retained at their addresses. A simple masking device has been designed that contains 200 $\mu$  spaces and 200 $\mu$  barriers, to allow each of 5 tetramers to couple to the solid support in distinct rows (Fig. 7). After addition of the first set of tetramers, the masking device is rotated 90°, and a second set of 5 tetramers are added. This can be compared to putting icing on a cake as rows, followed by icing as columns. The intersections between the rows and columns will contain more icing, likewise, each intersection will contain an octamer of unique sequence. Repeating this procedure for a total of 6 cycles generates 25 squares containing unique 24-mers, and the remaining squares containing common 12-mers (Fig. 10).



**Fig. 9.** Process for manufacturing an array (see following two pages for Fig. 10 and legends).

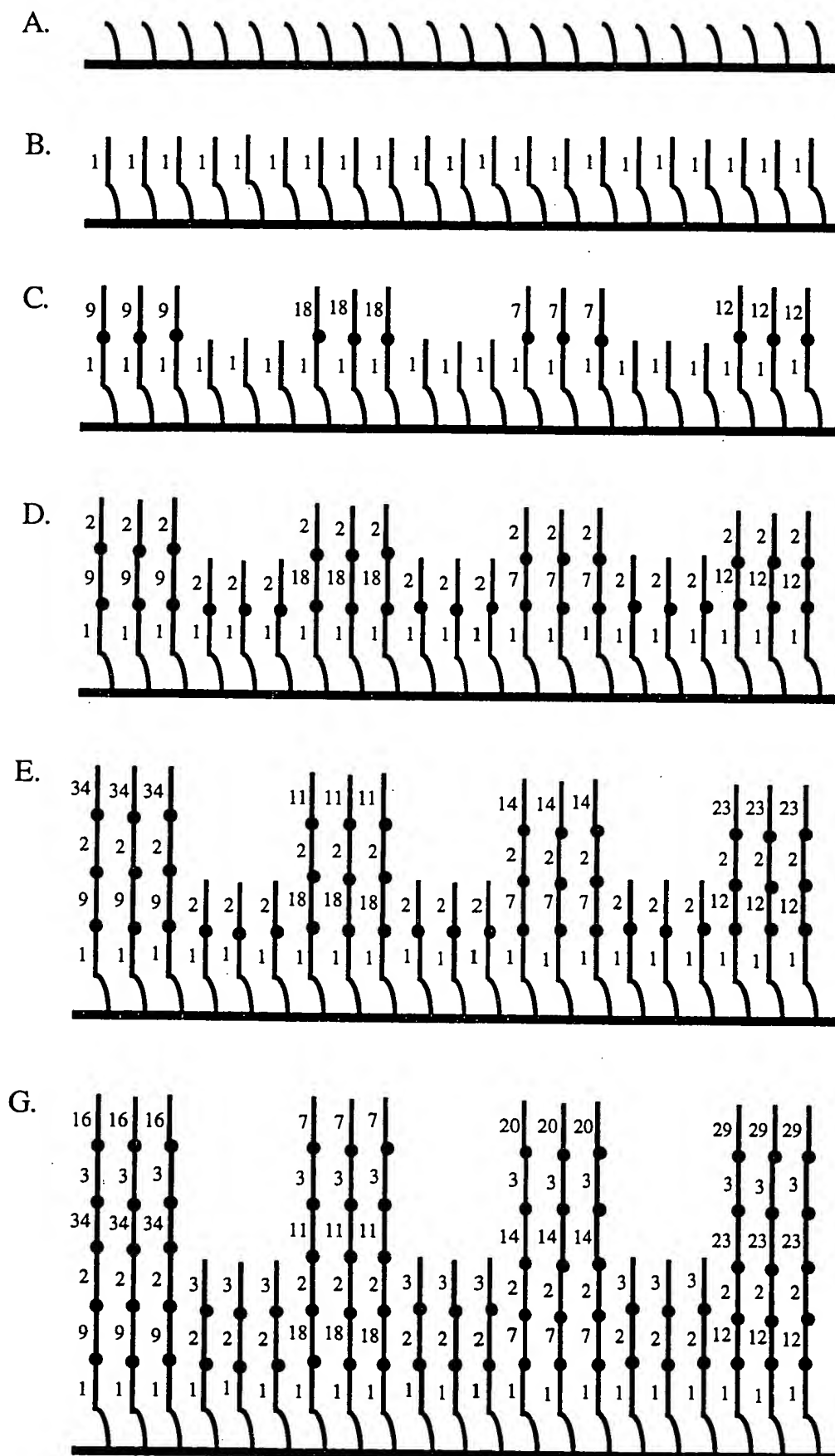


Fig. 10. Schematic cross-sectional view of synthesis of addressable array (see next page for legend).

**Fig. 9.** Process for manufacturing an array (legend). (A). Side view of reaction chambers. (B) Three-dimensional view of reaction chambers. Each wall and spacer is 100 $\mu$  thick. These spacers form chambers of width 100 $\mu$ . The multi-chamber device is pressed onto the membrane or activated solid surface, forming tight seals. The barriers may be coated with rubber or another material to avoid cross contamination from one chamber to the next. One must also make sure the membrane or solid support surface is properly wetted by the solvents. The membrane can be in a vertical position with a plate on each side to clamp the multi-chamber device to the membrane. Solvents are introduced at the bottom, rise up the chamber, and are removed from the top, much like an ABI four channel DNA synthesizer, except now there are 36 chambers and 36 different tetramer bottles. One proceeds by activating the surface, deprotecting, and adding a tetramer. The chamber is unclamped, the membrane is rotated 90°, and reclamped. A second round of tetramers are added. (C) Schematic bird's eye view of PNA oligomer array after completion of all 6 rounds of synthesis. Each tower represents 100 fmole of oligomers. Taller towers represent full size 24-mers which result from 6 rounds of synthesis in alternating directions. Each 24-mer tower represents a unique PNA sequence. Smaller towers represent half-size 12-mers which result from 3 rounds of synthesis in the same direction. All smaller towers in the same row as the arrows are of identical sequence. For clarity, the towers have been drawn as individual units, even though in the "real" synthesis they will be the same dimension as the grid squares and thus appear fused to each other. A "side view" of these individual towers is shown in Fig. 10.

**Fig. 10.** Schematic cross-sectional view of synthesis of addressable array (legend). (A) Attachment of flexible spacer (linker) to surface of array. (B) Synthesis of the first rows of PNA tetramers. Only the first row, containing tetramer 1, is visible. The multi-chamber device is placed so that additional rows, each containing a different tetramer, are behind the first row. (C) Synthesis of the first columns of PNA tetramers. The multi-chamber device has been rotated 90°. Tetramers 9, 18, 7, and 12 were added in adjacent chambers. (D) Second round synthesis of the PNA rows. The first row contains tetramer 2. (E) Second round of synthesis of PNA columns. Tetramers 34, 11, 14, and 23 were added in adjacent chambers during the second round. (F) (Not shown) Third round synthesis of PNA rows. The first row contains tetramer 3. (G) Structure of array after third round synthesis of columns, adding tetramers 16, 7, 20, 29. Note that all 24-mer PNA oligomers within a given row or column are unique, hence achieving the desired addressable array. Since each 24-mer differs from its neighbor by three tetramers, and tetramer differs from each other by at least 2 bases, then each 24-mer differs from the next by at least 6 bases. Each mismatch significantly lowers  $T_m$ , and the presence of 6 mismatches in just 24 bases would make cross hybridization unlikely even at 35°C. Note that the smaller 12-mer sequences are identical with one another, but are not at all common with the 24-mer sequences. Even though the particular 12-mer sequence may be found within a 24-mer elsewhere on the grid, for example 17-1-2-3-28-5, a zip code will not hybridize to the 12-mer at temperatures above 50°C.

Our design for a masking device is essentially the same as the masking technique developed by Maskos and Southern [33, 36]. This device will facilitate the desired array synthesis, and allow us to move on to testing zip code hybridization with Core B. The masking device will be designed and prepared by our industrial collaborators Dr. Ronald Cook of Siris Labs, and Dr. James Coull of Millipore. Simultaneously, we will also create, by hand, test arrays on membranes with aid of the Biorad dot blot apparatus containing individual microtiter wells.

## E. PROGRAM ASPECTS

We are developing solid-phase methodology which will allow multiplex detection of oligonucleotide ligation products that are indicative of cancer mutations. Specific aims of this project (Project 5) are: (i) Development and evaluation of solid support materials compatible with chemical synthesis of DNA oligonucleotides and PNA oligomers, and compatible with subsequent hybridization reactions. (ii) Establishment of methodology for synthesis of spatially addressable arrays of DNA oligonucleotides and PNA oligomers. (iii) Demonstration of scope and limitations of zip code concepts.

The zip code approach, including the key needs for its experimental implementation, arose through extensive discussions between F. Barany, R.P. Hammer, and G. Barany. The studies described in Project 5 that are directed towards solid support development and evaluation, with respect to either immobilization of pre-synthesized oligomers or their direct synthesis, will interface closely with efforts of Core B to prepare the needed DNA and PNA primer, zip code, and complementary zip code sequences, and to carry out hybridization assays. A collaboration with Project 3 will provide 5-propynyl-uridine monomers for incorporation into either DNA or PNA that may have improved thermodynamic parameters in hybridization. Progress in Project 5 on the preparation and application of spatially addressable arrays to detection of LDR products will have an immediate impact on the cancer work described by Project 1 and 2, since it will then

be possible to test clinical samples at a significantly enhanced throughput. We also anticipate considerable interactions between Project 5 and Core A for computer-aided design of zip code sequences.

Addressable array capture will eventually be the preferred method of identifying mutations. In our initial examination of the p53 gene in colon, lung, and breast tumor samples we will only look for nine different mutations, V157, R175, H179, C242, G245, R248, R249, R273 and R282 (See Projects 1 and 2). By synthesizing LDR primers with tails of varying lengths we can easily distinguish between these mutations using gel or capillary electrophoresis. However, increasing the number of assayable mutations eventually makes electrophoretic detection less feasible. This is due to two reasons. First, mutation-specific LDR primers should differ in length by two bases for their products to be distinguished by electrophoresis. For a large number of mutations to be assayed together, very long primers would have to be synthesized. Second, only one mutant signal would be expected for most reactions. Since mutant signals will differ from each other by only two bases, minor defects in a gel lane could cause a misreading of the LDR product length and incorrect identification of the mutation. Both of these problems are currently overcome in our laboratory by using more than one color fluorescent label and internal standards within the same lane. However, once the primers become very long (75-100 bases), failure sequences (n-1, n-2) become increasingly harder to separate by HPLC or gel purification. In contrast, by synthesizing a unique 24 base zip code sequence to each LDR primer, the product can be captured by its complementary zip code at a discrete "address" on a two-dimensional array. Failure sequences do not present a problem for either the zip code sequence or its complementary address. A fluorescent signal at a specific address, as opposed to a specific size, thus indicates the presence of a specific cancer mutation. (See Core B.)

A reusable addressable array with high capacity and excellent signal to noise specificity would be of benefit to several of our collaborators who need to detect large number of mutations. This will aid in the detection and identification of: hundreds of microorganisms by identifying 16s polymorphisms (Dr. Carl Batt), dozens of  $\beta$ -lactamase mutations responsible for third generation  $\beta$ -lactam resistance (Dr. Patrice Courvalin), epidemiological studies based on HIV polymorphisms (Dr. Olen Kew), dozens of polymorphisms in the E6 and E7 genes of high risk HPV strains (Dr. Saul Silverstein), multiple germline mutations in single gene disorders (Dr. Eric Hoffman, Dr. Perry White, and Dr. Emily Winn-Deen), and multiple somatic mutations in tumor suppressor genes and oncogenes (Dr. John Kovach, Dr. Michael Osborne, Dr. Basil Rigas, Dr. John Sninsky, Dr. Mark Sobel, Dr. Steven Sommer, and Dr. Thierry Soussi). Please see letters of collaboration in the overview section of this program project grant.

## F. TIMETABLE

*General:* The various aims of this research will be pursued in parallel, with successful results in one arena providing impetus for progress on other aspects. The focus of Project 5 is the chemical synthesis of zip code DNA and PNA sequences, ideally in spatially addressable arrays, on appropriately optimized solid supports. As requisite materials and/or structures become available, they will be tested in relatively short order by Core 2. The list below follows a combination of descriptions in "Specific Aims" and in "Experimental Design and Methods."

### Task 1. Design and optimization of zip code/address duplexes.

- a. Synthesis of zip code sequences and their complements as DNA, followed by solution annealing studies. Months 1 to 12.
- b. Synthesis of zip code sequences and their complements as PNA, followed by solution annealing studies. Months 7 to 24.
- c. Synthesis of 5-propynyl-U monomers (Figures 3 and 4), synthetic incorporation into DNA and PNA, and solution annealing studies. Months 12 to 36.

### Task 2. Development and evaluation of solid support materials compatible with chemical synthesis of DNA oligonucleotides and PNA oligomers, and compatible with subsequent hybridization reactions.

- a. Studies with commercially available membranes and literature methods for immobilization of end-group modified DNA and PNA. Months 1 to 30.
- b. Studies with "shaved" beads, which will be used for solid-phase synthesis of DNA. Months 1 to 18.
- c. Modifications in our laboratories of surfaces, beads, or membranes with hydrophilic spacers such as heterobifunctional polyethylene glycol (PEG) and/or carbohydrates (see Figure 6 and accompanying discussion), and further studies. Months 12 to 48.
- d. Development of novel chemistry for covalent immobilization of synthetic DNA or PNA (Figure 7). Months 6 to 30.
- e. Preparation and segment condensation of protected PNA tetramer building blocks (including optimization of protection scheme and coupling conditions), to build up 24-mer complementary zip code sequences which will be released into solution following chain assembly. Months 12 to 48.
- f. Segment condensation using protected PNA tetramer building blocks to build up 24-mer complementary zip code sequences which will be deprotected but retained on suitable solid supports for subsequent hybridization reactions. Months 36 to 60.

**Task 3. Establishment of methodology for synthesis of spatially addressable arrays of DNA oligonucleotides and PNA oligomers.**

- a. Adaptation of commercially available membranes and literature methods for immobilization of end-group modified DNA and PNA, in tandem with spot methods and/or masking technology, to prepare and test relatively small arrays. Months 12 to 48.
- b. Application of additional advances from Task 2 towards generation of spatially addressable arrays (e.g., "gluing" of "shaved" beads to solid surfaces, direct masking/segment condensation on solid supports as outlined in Figures 9 and 10). Months 12 to 60.

**G. HUMAN SUBJECTS / VERTEBRATE ANIMALS:** Not applicable

**I. CONSULTANTS/COLLABORATORS:** Project 5 represents a collaboration of Dr. George Barany (Principal Investigator), University of Minnesota, Dr. Robert Hammer, Louisiana State University, and Dr. Francis Barany, Cornell University, Medical College. In addition, we have excellent connections with leading industrial laboratories that are at the forefront of developing and commercializing methodologies for preparation of PEG-PS supports and functionalized membranes, PNA synthesis, and oligopolymer array construction. Specifically, we are collaborating with the team at Millipore led by Dr. James Coull, and a start-up company named Siris that is headed by Dr. Ronald Cook (see supporting letters). Recently, Dr. Michael Egholm, first author of several of the seminal papers on PNA, joined Millipore as a research chemist. Dr. Derek Hudson, a long-time collaborator of Dr. George Barany (several joint publications), is currently at Siris. Letters and Biographical Sketches for collaborators are attached in the overview section of this program project grant.

**J. CONSORTIUM/CONTRACTUAL ARRANGEMENTS:** Please see following page.

**CORNELL UNIVERSITY MEDICAL COLLEGE**  
**DEPARTMENT OF MICROBIOLOGY**

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**STATEMENT OF INTENT TO ESTABLISH A CONSORTIUM AGREEMENT**

**Date:** January 26, 1994

**Grant Number:** P01-

**P-01 Application Title:** PROGRAM PROJECT: NEW METHODS FOR  
CANCER DETECTION

Project # 5; DESIGN AND SYNTHESIS OF DNA AND  
PNA ARRAYS.

**Proposed Project Period:** Year 01; 12/01/94- 11/30/95

"The appropriate programmatic and administrative personnel of each institution involved in this grant application are aware of the NIH consortium grant policy and will establish the necessary inter-institutional agreement(s) consistent with that policy."

**CORNELL UNIVERSITY MEDICAL  
COLLEGE, NEW YORK, NY**

(Applicant Institution)

**UNIVERSITY OF MINNESOTA  
MINNEAPOLIS, MN.**

(Consortium Institution)

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Principal Investigator:

**FRANCIS BARANY, Ph.D.**

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Co-Investigator:

**GEORGE BARANY, Ph.D.**

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(name) (date)  
Official Authorized to Sign for Institution

**GREGORY W. SISKIND, M.D.**  
**ASSOCIATE DEAN**

---

(name) (date)  
Official Authorized to Sign for Institution

**K. LITERATURE CITED.**

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## **Core A.**

# **Informatic Support For Cancer Detection Methods**

**Core Leader: Neil R. Hackett  
Cornell University Medical College**

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. **DO NOT EXCEED THE SPACE PROVIDED.**

The goal of this Program Project is to develop techniques that detect multiple cancer mutations, ultimately for the purpose of researching the relationship between genetic alterations and tumor behavior, and applying these techniques in clinical situations. Managing a database of cancer-associated mutations, developing multiplex assays for them and correlating multiple cancer mutations with disease outcomes will require a sophisticated level of data management.

Core A will provide informatics support for cancer detection according to the following aims: (i). Create and maintain a database of mutations associated with cancer, patient history and experimental results. A relational Client/Server database will be created on a central facility consisting of a SPARC station 10 running the Sybase database management system. Both published reports and results from Projects 1 and 2 will be collected. (ii). Analyze database for correlations of point mutations with clinical outcome. The significance of the cancer detection experiments in Projects 1 and 2 will be assessed by performing multivariate analysis on given mutations to determine whether they predict clinical outcome. (iii) Write programs for the choice of primers for PCR/LDR, LDR/PCR and PCR/RE/LDR protocols. The programs will be written in C language for use on IBM/PC or Macintosh computers with a simple text-base interface. These programs will aid primer design and calculation of modified primer  $T_m$  values for Projects 1, 2 and 3. (iv). Assist in the design and analysis of oligonucleotide arrays for mutation detection. For project 5, potential schemes for array design will be explored to ensure arrays of the maximum difference in sequence between every pair of zip codes while maintaining a constant melting temperature. (v). Assist in the programming of the instruments in the diagnostics and evaluation core and interface these with the central database (for Core B). The informatics core will maintain a Client/Server database on a SPARC station, and provide programming support which is accessible to all participants in the program project. This research may lead to correlations between molecular markers and prognosis for lung, colon, breast, and cervical cancers.

PERSONNEL ENGAGED ON PROJECT, INCLUDING CONSULTANTS/COLLABORATORS. Use continuation pages as needed to provide the required information in the format shown below on *all* individuals participating in the project.

Name	<u>HACKETT Neil R.</u>	Degree(s)	<u>Ph.D.</u>	Social Security #	<u>REDACTED</u>
Position Title	<u>Assistant Professor</u>	D.O.B.	<u>REDACTED</u>	Role on Project	<u>Project leader.</u>
Organization	<u>Cornell University Medical College</u>			Department	<u>Microbiology</u>
Name	<u>GILES Aaron</u>	Degree(s)	<u>B.S.</u>	Social Security #	<u>REDACTED</u>
Position Title	<u>Programmer</u>	D.O.B.	<u>REDACTED</u>	Role on Project	<u>Programmer</u>
Organization	<u>Cornell University Medical College</u>			Department	<u>Acad. Computing</u>
Name		Degree(s)		Social Security #	
Position Title		D.O.B.		Role on Project	
Organization				Department	
Name		Degree(s)		Social Security #	
Position Title		D.O.B.		Role on Project	
Organization				Department	
Name		Degree(s)		Social Security #	
Position Title		D.O.B.		Role on Project	
Organization				Department	
Name		Degree(s)		Social Security #	
Position Title		D.O.B.		Role on Project	
Organization				Department	

P

PERSONNEL (Applicant Organization Only)				DOLLAR AMOUNT REQUESTED (omit cents)			
NAME	ROLE ON PROJECT	TYPE APPT. (months)	% EFFORT ON PROJ.	INST. BASE SALARY	SALARY REQUESTED	FRINGE BENEFITS	TOTALS
Neil R. Hackett	Project leader	12	20				
Aaron Giles	Programmer	12	100				
CORE A							
SUBTOTALS					\$53,400	\$17,088	\$70,488
CONSULTANT COSTS							\$0
EQUIPMENT (Itemize)							
SPARCstation 10, 41GX							
64Mb RAM, 1.3 Gb disk							\$15,000
SUPPLIES (Itemize by category)							
Operating system \$5,000							
Database \$7,000							
Compilers \$3,500							
Statistical \$1,500							\$16,500
TRAVEL							
One trip per year for P.I. to present results \$1,200							\$1,200
PATIENT CARE COSTS		INPATIENT					\$0
		OUTPATIENT					\$0
ALTERATIONS AND RENOVATIONS (Itemize by category)							\$0
OTHER EXPENSES (Itemize by category)							
Repairs \$2,500							\$2,500
SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD							\$105,688
CONSORTIUM/CONTRACTUAL COSTS							
DIRECT COSTS							\$0
INDIRECT COSTS							\$0
TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD (Item 7a, Face Page)							\$105,688

**BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD  
DIRECT COSTS ONLY**

CORE A

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED			
			2nd	3rd	4th	5th
<b>PERSONNEL:</b>						
<i>Salary &amp; fringe benefits</i>						
<i>Applicant organization only</i>		\$70,488	\$73,308	\$76,240	\$79,290	\$82,462
<b>CONSULTANT COSTS</b>		\$0	\$0	\$0	\$0	\$0
<b>EQUIPMENT</b>		\$15,000	\$2,000	\$2,000	\$2,000	\$2,000
<b>SUPPLIES</b>		\$16,500	\$17,160	\$17,846	\$18,560	\$19,302
<b>TRAVEL</b>		\$1,200	\$1,248	\$1,298	\$1,350	\$1,404
<b>PATIENT CARE COSTS</b>	INPATIENT	\$0	\$0	\$0	\$0	\$0
	OUTPATIENT	\$0	\$0	\$0	\$0	\$0
<b>ALTERATIONS AND RENOVATIONS</b>		\$0	\$0	\$0	\$0	\$0
<b>OTHER EXPENSES</b>		\$2,500	\$2,600	\$2,704	\$2,812	\$2,924
<b>SUBTOTAL DIRECT COSTS</b>		\$105,688	\$96,316	\$100,088	\$104,012	\$108,092
<b>CONSORTIUM/ CONTRACTUAL COSTS</b>		\$0	\$0	\$0	\$0	\$0
<b>TOTAL DIRECT COSTS</b>		\$105,688	\$96,316	\$100,088	\$104,012	\$108,092
<b>TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD</b>						<b>\$514,196</b>

JUSTIFICATION (Use continuation pages if necessary):

**From Budget for Initial Period:** Describe the specific functions of the personnel, collaborators, and consultants and identify individuals with appointments that are less than full time for a specific period of the year, including VA appointments.

**For All Years:** Explain and justify purchase of major equipment, unusual supplies requests, patient care costs, alterations and renovations, tuition remission, and donor/volunteer costs.

**From Budget for Entire Period:** Identify with an asterisk (\*) on this page and justify any significant increase or decrease in any category over the initial budget period. Describe any change in effort of personnel.

**For Competing Continuation Applications:** Justify any significant increases or decreases in any category over the current level of support.

**Equipment**

To achieve the aims of the informatics core, a high performance, multi-user server is essential. A networked SPARC station can simultaneously support multiple remote sessions of database access, primer design and interaction with equipment. The model requested maximizes computing power but provides no unnecessary graphics capacity or peripherals since we already have these features on the existing SPARCstation 2. The latter can not be used for the cancer detection project since it serves the whole campus as a DNA sequence analysis system, often using all its memory and cpu time for complex searches.

**Personnel**

Cornell University Medical College fringe benefits from 12/1/94 to 11/30/95 are at 32%. Salary increases of 4% are in accordance with both NIH and Cornell University Medical College guidelines. Salary

for Dr. Neil Hackett is comensurate with a 20% effort on this project. Dr. Hackett already single-handedly manages the DNA and Protein Sequence Analysis Facility of the Department of Microbiology.

The specific aims require the services of a programmer expert in C/C++ and design of graphic interfaces. Aaron Giles has already been hired by the Office of Academic Computing, and will be joining our efforts full time. He has the required expertise to write, test and document the programs described in the research description. His work will be supervised and assisted by the Core leader who anticipates a lot of time coordinating with the other investiagtors to establish exact requirements and experimental approaches that would benefit from informatic support.

### **Supplies**

The only supplies needed are software products to write and support the applications listed. We plan to take fully supported versions of the Software directly from SUNsoft to maximize the level of product support that is available for the relatively advanced applications we are planning. We also require a full performance DBMS and applications software such as that of Sybase, and a statistical analysis package. Software licences need yearly renewal and updates.



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**RESOURCES AND ENVIRONMENT**

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**FACILITIES:** Mark the facilities to be used at each performance site listed in Item 9, Face Page, and briefly indicate their capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Use "Other" to describe the facilities at any other performance sites listed in Item 9 on the Face Page and at sites for field studies. Use continuation pages if necessary. Include an explanation of any consortium/contractual arrangements with other organizations.

☐ Laboratory:

☐ Clinical:

☐ Animal:

☒ Computer:

A SPARCstation 2 with 32Mbyte memory and 2.5 Gbytes of storage is available, but is currently running the Genetics Computer Group DNA sequence analysis programs. The peripherals such as printer, tape and CD drives will be used with the SPARCstation 10 requested here. Co-investigators all have several PCs or MACs connected to the Internet.

☒ Office:

Office space for PI and programmer is available.

☐ Other ( ): \_\_\_\_\_

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**MAJOR EQUIPMENT:** List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

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**ADDITIONAL INFORMATION:** Provide any other information describing the environment for the project. Identify support services such as consultant, secretarial, machine shop, and electronics shop, and the extent to which they will be available to the project.

The support of the Office of Academic Computing is available for many aspects of this project, especially use of the network. The CUMC Clinical Research Center is contemplating a number of projects requiring similar informatics support and we will cooperate with programmers there.

## A. SPECIFIC AIMS:

(i). **Create and maintain a database of mutations associated with cancer, patient history and experimental results.** A relational Client/Server database will be created on a central facility consisting of a SPARC station 10 running the Sybase database management system. Three types of data will be collected: published reports on correlations of mutations with cancers; clinical data on patients for whom biopsies are available; and the results of experiments to search for mutations in cancerous and normal tissue performed in projects 1 and 2.

(ii). **Analyze database for correlations of point mutations with clinical outcome.** The significance of the cancer detection experiments in projects 1 and 2 will be assessed by performing multivariate analysis on given mutations to determine whether they predict clinical outcome.

(iii). **Write programs for the choice of primers for PCR/LDR, LDR/PCR and PCR/RE/LDR protocols.** The design of primers for the methods described in this proposal serves Projects 1, 2, and 4. The programs will be written in C for PCs and MACs with a simple text-based interface. They will be distributed to the participating sites and comments will be solicited for improvements. Sufficient flexibility will be written into the programs so that the stacking and base-pairing interactions of novel nucleotide analogs (see Project 3) can be input via a table of data independent of the executable program.

(iv). **Assist in the design and analysis of oligonucleotide arrays for mutation detection.** For project 5, potential schemes for array design will be explored to ensure arrays of the maximum difference in sequence between every pair of zip codes while maintaining a constant melting temperature. Programs for interpreting the output of the Fluorimager will also be written.

(v). **Assist in the programming of the instruments in the diagnostics and evaluation core and interface these with the central database.** The equipment of the Instrumentation and Mutation Detection core (DNA synthesizer, DNA sequencer, Fluorimager, and Biomek) will be networked to the central database (specific aim 1). The objective is to track reagents and experiments, eliminate redundancy and provide current information in a format available to all participants in the project.

## B. FACILITIES AND SKILLS OF THE INFORMATICS CORE

The informatics core will consist of a SPARC station 10 model 41GX running the Solaris 2.3 operating system to be located in the Department of Microbiology. This will be a part of a small computing facility managed by the Director of the Informatics core which currently consists of a Phosphorimager, Lynx5000 imaging system and a SPARC station 2. The last machine is connected to the Internet and has been successfully used to run remote sessions using the X11 interface. It is currently acting as a server for the analysis of protein and DNA sequence data for the whole Cornell University Medical Center campus.

The specific roles to be played by the informatics facility will require additional software. Specifically, we will require a commercial database system. A distributed, relational database running in Client/Server mode will be purchased. Based on the experience of the Clinical Research Centers at both CUMC and NYU, we believe that Sybase will be the most suitable system. This software provides both the Database Management System (DBMS) and the Database Application software for providing a coherent interface to the users. Sybase is also capable of handling distributed data which may be useful since a clinical database is already established at the Strang Cancer Prevention Center. In addition we will need a C/C++ compiler and X11 developers kit in order to write specific programs such as the LDR primer design program. A comprehensive statistical program such as the advanced versions of Statistical Package for Social Sciences (SPSS) will also be needed.

The facility will be staffed by a full-time programmer/system manager, Aaron Giles (see Key Personnel). This individual will create, optimize and maintain the database, write programs, administer the system and educate personnel in use of the facility. He will also be responsible to install communications with remote sites so they have equal access to the facility as those already at Cornell Medical College. The work of

the programmer will be directed by Neil R. Hackett, currently Manager of the DNA and Protein Sequence Analysis Facility of the Department of Microbiology. Priorities will be set in consultation with other project leaders according to the distribution of usage in the Table of Distribution of Core Facilities. The clinical/molecular database will be designed with the help of Dr. Matthew Lubin of the Strang Cancer Prevention Center.

## C. ROLES OF THE INFORMATICS CORE

(i). **Create and maintain a database of mutations associated with cancer, patient history and experimental results.** A relational Client/Server database will be created on a central facility consisting of a SPARC station 10 running the Sybase database management system. Three types of data will be collected: published reports on correlations of mutations with cancers; clinical data on patients for whom biopsies are available; and the results of experiments to search for mutations in cancerous and normal tissue performed in projects 1 and 2.

Each type of data is partially related to the others. Hypothetically, we might begin with a published report that a particular point mutation in the p53 gene is associated with a subset of breast cancer. This mutation would be entered into a directory of cancer-associated mutations and a suitable experimental protocol will be designed (see Informatic Core Specific aims 3 & 4). The feasibility of the experimental design would be proven on purified DNA bearing the mutation of interest. At that point the status of the p53 mutation in the mutation directory will be modified to "detectable". We would search the clinical database for patients who fit the clinical description of the original report and for whom biopsy samples are available. An experimental determination of the status of these patient biopsies for that mutation would be run. The experimental data would then be subjected to a multivariate analysis in combination with other known mutations for those particular biopsies to assess predictors of clinical outcome. The existence of an apparent correlation would prompt further studies until that correlation is proven to be statistically significant. At that point, the status of the p53 mutation in the database would be modified to being an "informative".

There are three external sources of data for the database: Public databases of mutations correlated with cancer; the Strang Cancer Prevention Center clinical database and the experimental results obtained from Project 1 (Lung and Colon Cancer) and Project 2 (Breast and Cervical Cancer).

The OMIM database and the mutation manager subsection of the GDB database contain comprehensive lists of the germline mutations known in all genes. It is the policy of these databases to keep these data up to date through investigator submissions and literature searches. These databases are accessible through the internet and simple search routines are possible. Presently the searches need to be done manually using the date field of the record to find newly added information. GDB is currently writing routines that will allow only new acquisitions to be collected. Therefore we are sure that we will be able to simply obtain one of the required informatic aspects, that is a list of point mutations in oncogenes. Both GDB and OMIM allow correlation with the original citation and the accession number in GenBank. Some somatic mutations are available through OMIM and GDB. Others are available through databases maintained by individual labs which we would access through internet. For example, Drs. John Kovach and Steven Sommer of the Mayo Clinic, and Dr. Thierry Soussi of the Institute de Genetique Moleculaire have maintained databases of over 2,000 mutations found in the p53 gene in various cancers. In addition, we will set up scripts to periodically search literature databases (eg. Medline or Science Citation Index) for combinations of keywords.

The second kind of data to be collected is case histories for the patients whose biopsies are to be analyzed experimentally. In particular, we are interested in following the status of these patients over time. Dr. Michael Osborne, Director of the Strang Cancer Prevention Center, has collected the biopsies to be analyzed and has a small database of patient histories corresponding to many of the available samples (See letter of Collaboration. These will be translated to Sybase format with the identity of the patient encoded. Where no digital record is available, the data will be entered manually.

This rather crude database should be sufficient for simple correlation of well-defined clinical outcomes (eg. regression, cure, recurrence) with molecular markers. In the future, more complex studies involving

large numbers of patients will be performed requiring a more sophisticated database with full interconnectivity to the computer system at Strang. This is beyond the scope of this core facility but there are efforts by Dr. Steven Erde of Cornell Medical College to implement such an interconnection which we would immediately use once available.

The third type of data is laboratory data. The raw data will primarily consist of traces from the ABI Automatic Sequencer which will be processed by the ABI sequencer to sizes and intensities of peaks. These in turn will be interpreted in terms of the presence, absence or relative level of a series of mutations. This data will be exported to the central database along with experimental details about its source. The DBMS will ensure that data can only be imported when all the required links have been put in place (protocol, primers, biopsy ID, etc). These links correspond to other lists in the central database such as the primer list or protocol list.

Maintenance of such data is best achieved by a relational database. A series of cross-referenced tables will be designed to contain all anticipated types of information. One major advantage of the relational model is that unanticipated features, which we subsequently decide are important, can easily be added to one of the tables. There are also important issues of database integrity that can be handled by a high-performance database. For example, a large number of laboratory experiments are likely to be performed which the researchers will enter into the database. The DBMS can be programmed to accept these data and search for correlations only when a complete set of experimental parameters such as primer used, exact conditions and times etc. have been provided. Finally, the DBMS can prevent access to any confidential data by unauthorized users.

A high-end DBMS such as Sybase provides the database application software that drives sessions for users at distant sites. Thus, authorized investigators at Strang or other project sites will be able to log into the SPARC station and use the database. Sybase also provides the Structured Query Language to allow users to frame questions of the data to develop hypotheses of their own.

**(ii). Analyze database for correlations of point mutations with clinical outcome.** The significance of the cancer detection experiments in projects 1 and 2 will be assessed by performing multivariate analysis on given mutations to determine whether they predict clinical outcome.

An investigation of correlation will begin with a hypothesis based on publications or communications from other investigators. The information flow is described in the preceding section. The Sybase Structured Query Language (SQL) can then be used to extract the relevant data from the central database and reformat it for the statistical analysis program SPSS. The expected outcome of many inquiries are weak associations of insufficient predictive value to be used clinically. But when a full multivariate analysis is performed we anticipate more striking correlations. When strong correlations are seen the informatics core will provide assistance in the design of a survey sufficiently large that a correlation can be determined with a high degree of statistical confidence.

**(iii). Write programs for the choice of primers for PCR/LDR, LDR/PCR and PCR/RE/LDR protocols.** The design of primers for the methods described in this proposal serves Projects 1, 2, and 4. The programs will be written in C for PCs and MACs with a simple text-based interface. They will be distributed to the participating sites and comments will be solicited for improvements. Sufficient flexibility will be written into the programs so that the stacking and base-pairing interactions of novel nucleotide analogs (see Project 3) can be input via a table of data independent of the executable program.

A number of programs have been written for designing PCR primers. The success of these indicates that we can reasonably calculate the expected hybridization behavior of oligonucleotides from base stacking energies and use this data to design primers for other protocols. The basic LDR primer design program is extremely simple. Three parameters need to be considered: (i), to detect more than one mutation on the same PCR product, all LDR primers have to be complementary to the same strand; (ii), the position to be ligated can be either immediately 5' or 3' to the mutation; (iii), the length of the oligonucleotide can be varied. All possibilities will be considered to give a set of oligonucleotides that should detect the required mutations and give a  $T_m$  of  $75^\circ\text{C}$  ( $\pm 2^\circ\text{C}$ ). A further weighting factor may be introduced subsequently when we find what

mismatches in both the 3' or 5' position give best discrimination between mutant and wild type. For a series of mutations the addition of appropriate zip code tails and fluorescent labels will be designed so that all products can be distinguished.

For this simple case, a program can reasonably consider all possible primers within user-specified limits. Primers for detection of somatic mutations require a different set of interdependent variables to be evaluated. Ultimately, we want to use a standard PCR/RE/LCR protocol to simultaneously detect a large number of possible mutations. Unlike the simple PCR/LDR protocol, this protocol needs to evaluate the effect of more parameters. This is a multivariate optimization problem that could be approached by a number of algorithms. It is clear however, that all possible combinations of parameters cannot be explored. The parameters to be varied include:

- (i) The restriction enzyme to use. Every point mutations is in four dinucleotides (two on each strand) - we can pick any one to go with an appropriate enzyme.
- (ii) Mismatch to be extended
- (iii) Base analogue to be used and efficiency of conversion to desired basepair
- (iv) Necessity for a thiophosphate in one or both oligonucleotides
- (v) The length and sequence of the oligonucleotide pairs.

A large number of possible oligonucleotide combinations will be evaluated to give an index of how effectively they might work. The quantitative measure of how they work will have to be refined on the basis of experimental data which will be used to feedback on the weighting given to various parameters in the design process. For example, preferred restriction enzymes and mismatches will emerge as being optimal for mutation detection.

We have also manually explored the process of designing an LDR/PCR protocol for detecting gene copy number. The procedure could readily be automated using shell scripts that access the GCG sequence analysis programs and a small number of new programs. Basically, we searched in the exons of the gene of interest (for example p53) for the sequence WCCW followed by any restriction site 5 to 25 nucleotides downstream. We then chose those sequences where the CC nucleotide corresponded to a proline in the protein on the premise that this made conservation between individuals most likely. We then further refined the list to those where the (G+C)-content of the flanking sequences was about 50% for a region corresponding to a primer with  $T_m$  of 75°C.

**(iv). Assist in the design and analysis of oligonucleotide arrays for product detection.** For project 5, potential schemes for array design will be explored to ensure arrays of the maximum difference in sequence between every pair of zip codes while maintaining a constant melting temperature. Programs for interpreting the output of the Fluorimager will also be written.

The design of PNA arrays is a combinatorial problem but the number of permutations is extremely high and cannot be systematically explored. We have simplified this problem by the design of tetramer sequences which contain the following features: (i) They differ from each other by at least two bases. (ii) Self complementary sequences are not used (iii) Palindromic or repetitive sequences are not used. Nevertheless, from a pool of 36 tetramers, 376,992 possible combinations of five different tetramers are possible. For making a five by five array of tetramers, by the method described in Project 5, there are  $5.4 \times 10^{16}$  possible combinations. We will write a program that explores a wide range of the decision surface beginning at arbitrary points and choosing on the basis of maximum difference, from the other 24 mers being built. Note that each zip code address will differ from its neighbor in either a row or column by three tetramer units, or at least 6 bases. Thus, an oligonucleotide hybridizing to a position in the addressable array at a  $T_m$  of, for example, 96°C, will have a  $T_m$  of at least 48°C less (-8°C for each PNA/DNA mismatch) to any neighboring position. The solutions will be evaluated on the basis that the oligonucleotides designed have comparable  $T_m$  values, have no predictable tendency to base pair with themselves, and have a maximum difference from all

other sequences in the array. In Project 5, the use of propynyl U instead of T will be explored for increasing the melting temperature contribution of A:T bases in the zip code array. If this nucleotide analogue does not increase  $T_m$  values, the program for designing the zip code array will place a greater emphasis on equalizing  $T_m$  values from one address to the next. The entire zip code array will be tested as described in Core B.

Reading arrays is a standard image processing problem. Using the Molecular Dynamics FluorImager 575 allows the required analysis to be performed automatically since a user-specified grid can be defined and manipulated and the intensity within each cell can be calculated and exported to a spreadsheet. Positive control spots are essential to achieve reliable location of the grid. The informatics core will assist in analysis of the exported spreadsheet and extracting the identity of the LDR product from the data. This will require an oligonucleotide database and writing programs that describe the experimental design and allow conversion of location to the identity of the mutation detected. These programs will be written in C++ as a database application to run on the SPARC station with the X11 interface.

**(v). Assist in the programming of the instruments in the diagnostics and evaluation core and interface these with the central database.** The equipment of the Instrumentation and Mutation Detection core (DNA synthesizer, DNA sequencer, Fluorimager, and Biomek) will be networked to the central database (specific aim 1). The objective is to track reagents and experiments, eliminate redundancy and provide current information in a format available to all participants in the project.

Existing tools are completely capable of performing the desired objectives. Two specific projects are clearly important. The first is to interface the instruments, especially the oligonucleotide synthesizer, with the central database so every synthesis has a unique identifier that allows us to track experiments and inventory. There is an RS232 port on the synthesizer and exporting the data to a computer can be a required step in any synthesis protocol.

Secondly, we will write programs for the Beckman Biomek or ABI Catalyst 800 robotic workstation describing the basic protocols (PCR/LDR, PCR/RE/LDR and LDR/PCR) and variations for specific applications. The Biotest language is available from Beckman which is a text-like language that can be converted into the GENESIS operating system that runs the Biomek. From flow charts describing the desired protocols we will build up full descriptions of the procedures and evaluate these on dummy reagents on the Biomek. Programs will be documented and a clear user interface with instructions about where every reagent should be placed will be a part of the project.

**D. Human Subjects / Vertebrate Animals:** Not applicable

**E. Consultants/Collaborators:** Letters and Biographical Sketches for collaborators are attached in the overview section of this program project grant.

**F. Consortium/Contractual Arrangements:** Please see overview.

## **Core B.**

### **Instrumentation and Mutation Detection**

**Core Leader: Francis Barany  
Cornell University Medical College**

**Core Co-Leader: Matthew Lubin  
Strang Cancer Prevention Center**

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. **DO NOT EXCEED THE SPACE PROVIDED.**

Correlations of multiple cancer mutations with disease outcome will require the ability to perform high throughput mutation detection. The goal of this core is to provide the instrumentation and mutation detection support required to achieve large scale identification and analysis of mutations. Core B will work closely with Core A, the informatics support for cancer detection methods.

This Core will have the following responsibilities: (i) Providing instrumentation for oligonucleotide synthesis and analysis of cancer causing mutations. The PCR/LDR, LDR/PCR, and PCR/RE/LDR experiments described in Projects 1 and 2 require synthesizing large numbers of oligonucleotides. The products from these cancer detection amplifications will be separated and quantified on an ABI 373A DNA sequencer. By the third year we plan to automate some of the PCR/RE/LDR steps using a robotics workstation. (ii) Testing the efficiency and polymerase fidelity of nucleotide conversions using convertide oligonucleotides. The PCR/RE/LDR cancer detection scheme has the potential of detecting cancer mutations at a sensitivity of 1 in  $10^6$  or  $10^7$ . The sensitivity of this scheme is dependent on the fidelity of thermostable polymerase extension off primers containing a 3' nucleotide analogue (Project 3). Using an assay we developed, the Core will test both the efficiency and fidelity of different polymerases for each base conversion. (iii) Testing oligonucleotide or PNA addressable arrays for quantitative cancer mutation detection. Large scale detection of a multitude of mutations will require addressable arrays. Mutations will be distinguished by the position of a fluorescent signal on the array. The Core will test arrays synthesized in Project 5 for fluorescent detection of LDR and LCR oligonucleotide products, using a Molecular Dynamics FluorImager 575.

PERSONNEL ENGAGED ON PROJECT, INCLUDING CONSULTANTS/COLLABORATORS. Use continuation pages as needed to provide the required information in the format shown below on *all* individuals participating in the project.

Name	<u>BARANY, Francis</u>	Degree(s)	<u>Ph.D.</u>	Social Security #	<u>REDACTED</u>
Position Title	<u>Associate Professor</u>	D.O.B.	<u>REDACTED</u>	Role on Project	<u>Prin. Investig.</u>
Organization	<u>Cornell Univeristy Medical College</u>			Department	<u>Microbiology</u>
Name	<u>LUBIN, Matthew</u>	Degree(s)	<u>M.D.</u>	Social Security #	<u>REDACTED</u>
Position Title	<u>Director of Medical Genetics</u>	D.O.B.	<u>REDACTED</u>	Role on Project	<u>Co-investigator</u>
Organization	<u>Strang Cancer Prevention Center</u>			Department	<u>Medical Genetics</u>
Name	<u>LUO, Jianying</u>	Degree(s)	<u>Ph.D.</u>	Social Security #	<u>REDACTED</u>
Position Title	<u>Research Associate</u>	D.O.B.	<u>REDACTED</u>	Role on Project	<u>REDACTED</u>
Organization	<u>Cornell Univeristy Medical College</u>			Department	<u>Microbiology</u>
Name	<u>LU, Jing</u>	Degree(s)	<u>B.A.</u>	Social Security #	<u>REDACTED</u>
Position Title	<u>Technician</u>	D.O.B.	<u>REDACTED</u>	Role on Project	<u>REDACTED</u>
Organization	<u>Cornell Univeristy Medical College</u>			Department	<u>Microbiology</u>
Name		Degree(s)		Social Security #	
Position Title		D.O.B.		Role on Project	
Organization				Department	
Name		Degree(s)		Social Security #	
Position Title		D.O.B.		Role on Project	
Organization				Department	
Name		Degree(s)		Social Security #	
Position Title		D.O.B.		Role on Project	
Organization				Department	



Principal Investigator/Program Director (Last, first, middle): **F. BARANY, Ph.D.**  
**DETAILED BUDGET FOR INITIAL BUDGET PERIOD**  
**DIRECT COSTS ONLY**

FROM 94/12/01 THROUGH 95/11/30

PERSONNEL (Applicant Organization Only)		TYPE APPT. (months)	% EFFORT ON PROJ.	INST. BASE SALARY	DOLLAR AMOUNT REQUESTED (omit cents)		
NAME	ROLE ON PROJECT				SALARY REQUESTED	FRINGE BENEFITS	TOTALS
Francis Barany	Principal Investigator	12	10				
Matthew Lubin	Co-Investigator	12	5				
Jianying Luo	Research Associate	12	50				
Jing Lu	Research Technician	6	100				
<b>CORE B</b>							
SUBTOTALS					\$45,938	\$14,700	\$60,638
CONSULTANT COSTS							
							\$0
EQUIPMENT (Itemize)							
FluorImager 575 \$80,100							\$80,100
SUPPLIES (Itemize by category)							
DNA modifying Enzymes: \$1,000							
Electrophoresis supplies \$3,000							
DNA Synthesis reagents \$7,000							\$11,000
TRAVEL							\$0
PATIENT CARE COSTS							\$0
INPATIENT							\$0
OUTPATIENT							\$0
ALTERATIONS AND RENOVATIONS (Itemize by category)							\$0
OTHER EXPENSES (Itemize by category)							
Equipment maintainance \$5,000							\$5,000
<b>SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD</b>							<b>\$156,738</b>
CONSORTIUM/CONTRACTUAL COSTS							
DIRECT COSTS \$0							TOTAL—> \$0
INDIRECT COSTS \$0							
<b>TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD</b> (Item 7a, Face Page) —>							<b>\$156,738</b>

**BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD  
DIRECT COSTS ONLY**

CORE B

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED			
			2nd	3rd	4th	5th
<b>PERSONNEL:</b>						
<i>Salary &amp; fringe benefits</i>						
<i>Applicant organization only</i>		\$60,638	\$68,199	\$70,927	\$73,764	\$76,715
<b>CONSULTANT COSTS</b>		\$0	\$0	\$0	\$0	\$0
<b>EQUIPMENT</b>		\$80,100	\$0	\$70,000	\$0	\$0
<b>SUPPLIES</b>		\$11,000	\$11,440	\$11,898	\$12,374	\$12,869
<b>TRAVEL</b>		\$0	\$0	\$0	\$0	\$0
<b>PATIENT CARE COSTS</b>	<b>INPATIENT</b>	\$0	\$0	\$0	\$0	\$0
	<b>OUTPATIENT</b>	\$0	\$0	\$0	\$0	\$0
<b>ALTERATIONS AND RENOVATIONS</b>		\$0	\$0	\$0	\$0	\$0
<b>OTHER EXPENSES</b>		\$5,000	\$5,200	\$5,408	\$5,624	\$5,849
<b>SUBTOTAL DIRECT COSTS</b>		\$156,738	\$84,839	\$158,233	\$91,762	\$95,433
<b>CONSORTIUM/ CONTRACTUAL COSTS</b>		\$0	\$0	\$0	\$0	\$0
<b>TOTAL DIRECT COSTS</b>		\$229,263	\$84,839	\$158,233	\$91,762	\$95,433
<b>TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD</b>						<b>(Item 8a)-&gt; \$659,530</b>

**JUSTIFICATION (Use continuation pages if necessary):**

**From Budget for Initial Period:** Describe the specific functions of the personnel, collaborators, and consultants and identify individuals with appointments that are less than full time for a specific period of the year, including VA appointments.

**For All Years:** Explain and justify purchase of major equipment, unusual supplies requests, patient care costs, alterations and renovations, tuition remission, and donor/volunteer costs.

**From Budget for Entire Period:** Identify with an asterisk (\*) on this page and justify any significant increase or decrease in any category over the initial budget period. Describe any change in effort of personnel.

**For Competing Continuation Applications:** Justify any significant increases or decreases in any category over the current level of support.

**Personnel:** Cornell University Medical College and Strang Cancer Prevention Center salaries are in accordance with the high cost of living in New York City, as well as the experience of the personnel. A 10% effort by the Principal Investigator and a 5% effort by Co-investigator assures full supervision of the junior personnel in this project. The Co-investigator will increase his effort and salary to 10% for years 2-5 reflecting increased responsibility in the project as our testing of breast biopsies expands. Cornell University Medical College has granted the Principal Investigator a Hirschl/Monique Weill-Caulier Career Scientist Award from 01/01/92 to 01/01/1997. This award of \$20,000 / year may be used as salary (and fringe benefit support) only. It thus allows the P.I. to spend full effort on research.

Dr. Jianying Luo is a research associate (Ph.D.) who has been in the Principal Investigator's laboratory since 9/01/92. Dr. Luo obtained her Ph.D. in the laboratory of Dr. Joseph Krakow at Hunter College. She is a highly skilled member of the Principal Investigator's laboratory. She has constructed,

isolated, sequenced, and characterized the proteins from over 30 site-specific mutants of the *Tth*. ligase gene. In addition, she has performed all the ligase fidelity assays described in the Project 4. This has required extensive use of our DNA sequencer, DNA synthesizer, and PCR machines. Ms. Jing Lu joined the P.I.'s laboratory in January of 1994 as a research technician. Her responsibility will be synthesis of oligonucleotides and pouring gels for the DNA sequencer. Dr. Luo has already taught Ms. Lu how to do this. Ms. Lu is the wife of Dr. Weguo Cao, who is a Research Associate in the P.I.'s laboratory. Although she has been hired on a part time basis, the P.I. has noted that she works on the weekends, as do the other members of the P.I.'s laboratory. The high cost of living in New York City necessitates competitive salaries.

*Fringe benefits:* Cornell University Medical College fringe benefits from 12/1/94 to 11/30/95 are at 32%. Salary increases of 4% are in accordance with Cornell University Medical College guidelines. Cornell University Medical College documentation of calculation of the indirect cost and fringe rate are attached.

*Equipment:* Our capacity to synthesize large numbers of oligonucleotides, perform large numbers of LCR, LDR, and PCR amplifications, and fluorescent quantification of LDR products on a DNA sequencer has been tremendously augmented by generous equipment gifts from Roche Molecular Diagnostics, Perkin Elmer, and Applied Biosystems Inc. Specifically, we now have our own PE 9600 thermal cycler, ABI 394 DNA synthesizer, and an ABI 373 automated DNA sequencer. Although these instruments were placed in my laboratory for use in developing the Ligase Chain Reaction (LCR) for detection of infectious and genetic diseases and isolating new thermophilic proteins, all three companies have given their encouragement to use these instruments for cancer research. Such equipment still necessitates highly skilled and motivated personnel to run them.

We are requesting a Molecular Dynamics Fluroimager 575 for the first year. This includes the FluorImager, a High resolution monitor, and an internal Magneto-optical disk drive with 650 MB capacity per disk. This is currently the only Fluroimager on the market, and it already has the capacity to detect about 2 attomoles of fluorescently labeled primer in a single 50 x 50 $\mu$  pixel. We will integrate the Fluroimager with our SPARCstation 10 and network all the users to this unit. If an upgraded machine becomes available or another company produces their own Fluroimager at the time of funding, we would use the most sensitive instrument available. The Fluroimager is essential for the reading cancer mutations captured by addressable arrays, designed and synthesized in Project 5.

We are also requesting a robotic work station in the third year. Sufficient progress will have been made by that time to justify large scale diagnostics. The Biomek (Beckman) Robot with Side Loader, Filtration assembly, Thermal Cycler and Incubator Stack Accessory costs from \$70,000 to \$90,000. The Biomek (Beckman) is currently the most versatile pipetting work station and is available with accessories for cold storage of large numbers of reagents. Currently, plates have to be carried from the Biomek to the thermal cycler. But an interface with a thermal cycler is being developed which would allow the more complex protocols such as PCR/RE/LDR to be performed without manual intervention on up to 96 samples at once. To evaluate a large number of different optimization efforts and to process even modest numbers of samples, the degree of reproducibility and lack of manual work afforded by such a system is essential. An alternative robotic workstation is the Catalyst 800 manufactured by ABI (about \$70,000 to \$80,000). This instrument has a single channel automated pipetor and built in Thermal Cycler. Although only partially programable this year, the machine should be substantially upgraded and fully programable by the third year of this project.

*Supplies.* We require some supplies for Core B. The most important items will be the DNA synthesis reagents and chemicals. Our current detection methods depend on separating LCR and LDR products on a sequencing gel. This requires a large number of sequencing gels. Our DNA modifying enzymes include restriction enzymes (we make our own *TaqI*), T4 kinase, T4 Ligase, Klenow, *Taq* Polymerase, and other enzymes.

*Other Expenses.* We estimate equipment maintainance and repair costs to be about \$5,000 for the first year.

**RESOURCES AND ENVIRONMENT**

**FACILITIES:** Mark the facilities to be used at each performance site listed in Item 9, Face Page, and briefly indicate their capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Use "Other" to describe the facilities at any other performance sites listed in Item 9 on the Face Page and at sites for field studies. Use continuation pages if necessary. Include an explanation of any consortium/contractual arrangements with other organizations.

☒ **Laboratory:** The Barany group currently numbers 6 full time researchers including the P.I., and one part time technician. The lab is on the fourth floor of the microbiology wing, and comprises 3,500 sq. ft. of relatively new space (8 yrs since renovation.) The Barany lab is 670 sq. ft., in addition, a cold room, an equipment room, a dark room and a computer room are shared with Dr. William Holloman.

☐ **Clinical:**

☐ **Animal:**

☒ **Computer:** The P.I. has a Macintosh Quadra 840 AV (in his office), four Macintosh IICI, and one Macintosh Classic computer which is used primarily to program our HPLC. Our Microbiology Dept. has a SPARCstation 2 with the Genetics Computer Group package of programs for retrieval and analysis of protein and DNA sequences. We are also directly connected with the Rockefeller University computer.

☒ **Office:** The P.I. has a private office of about 180 sq. feet.

☐ **Other ( ):**

**MAJOR EQUIPMENT:** List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

With Dr. Holloman, the P.I. share one ultracentrifuge, 2 high speed and 2 low speed centrifuges, several microfuges, a liquid scintillation counter, 2 Vis/UV spectrophotometers (one with kinetics), a fluorescence spectrophotometer, 2 PE thermal cyclers, an FPLC, an HPLC, 3 ultralow freezers, a fermenter, 2 floor shakers, 2 chemical hoods, a biological hood, a french press, a sonicator, a lyophilizer, a speed vac, a rotary evaporator, several water bath shakers, fraction collectors, gel dryers, power supplies, freezers, refrigerators, and incubators. Our department has a phosphorimager which is shared among our faculty. In addition, Roche, PE, and ABI, have placed a PE 9600 thermal cycler, an ABI 394 DNA synthesizer, and an ABI 373 automated DNA sequencer in my laboratory with encouragement to use these instruments for research on the Cancer detection studies, as well as for the other projects.

**ADDITIONAL INFORMATION:** Provide any other information describing the environment for the project. Identify support services such as consultant, secretarial, machine shop, and electronics shop, and the extent to which they will be available to the project.

Our department of Microbiology has grown to 7 members under the leadership of Dr. Kenneth I. Berns. He has forged a joint Graduate program in Molecular Biology with Dr. Jerry Hurwitz at our neighboring Sloan Kettering Memorial Research Institute, so our joint department numbers 30 faculty members. We have full access to the core facilities at Sloan Kettering. Dr. Neil Hackett is within our department, and Dr. Mathew Lubin is At the Strang Cancer Cancer Prevention center across the street. In addition, I have an adjunct appointment at The Rockefeller University (also across the street) in the Department of Chemistry, Biochemistry, and Structural Biology, now headed Dr. David Cowburn. Finally, my X-ray collaborator, Dr. Aneel Aggarwal is a few miles uptown at the College of Physicians & Surgeons, Columbia University. Thus, we can all easily get together to discuss new results and ideas.

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**RESOURCES AND ENVIRONMENT**

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**FACILITIES:** Mark the facilities to be used at each performance site listed in Item 9, Face Page, and briefly indicate their capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Use "Other" to describe the facilities at any other performance sites listed in Item 9 on the Face Page and at sites for field studies. Use continuation pages if necessary. Include an explanation of any consortium/contractual arrangements with other organizations.

☒ **Laboratory:** The Strang-Cornell laboratory space totalling about 4500 Square feet is located at 510 East 73rd Street, a short walk from the Strang Cancer Prevention Center's offices and the Cornell Medical Center. The facility includes a cold room and tissue culture room with laminar flow hood. Approximately 320 square feet are dedicated for use by Dr. Lubin.

☒ **Clinical:** The Strang Cancer Prevention Center has two main resources for clinical investigations. Patient visits at Strang come to more than 5000 per year. Of these approximately 4000 are for breast cancer screening, preoperative and follow up evaluations for breast cancer surgery, and breast cancer chemotherapy. The Strang National Registry for women with family histories of breast cancer maintains and updates family history information on over 14,000 women. All clinical investigations at Strang are approved by Strang's IRB. We also enjoy the close cooperation of the Department of Pathology and the Division of Oncology at the New York Hospital-Cornell Medical Center.

☐ **Animal:**

☒ **Computer:** Dr. Lubin's laboratory is equipped with a Macintosh SE/30 computer and StyleWriter printer. In the office he has a Macintosh IIfx with a LaserWriter printer and a PowerBook 140.

☒ **Office:** Dr. Lubin's office is located at the Strang Cancer Prevention Center at 428 East 72nd Street. His office space amounts to approximately 150 square feet.

☐ **Other ( ):**

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**MAJOR EQUIPMENT:** List the most important equipment items already available for this project, noting the location and pertinent capabilities of each. Equipment includes: Ultracentrifuge (Becman, L8-70m). UV-Visible spectrophotometer (Perkin-Elmer). Beta counter (Pacard Tricarb 300). Thermal Cycler (Perkin-Elmer 9600). Speed-Vac concentrator. Gamma counter (LKB). Freezers -20 and -70 degrees. Tissue culture facilities.

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**ADDITIONAL INFORMATION:** Provide any other information describing the environment for the project. Identify support services such as consultant, secretarial, machine shop, and electronics shop, and the extent to which they will be available to the project.

The Strang Cancer Prevention Center is across the street from Cornell University Medical College and the New York Hospital. This close proximity facilitates the collaboration between the P.I. and Co-I.

**A. SPECIFIC AIMS:**

(i) **Provide instrumentation for oligonucleotide synthesis and analysis of cancer causing mutations.** The PCR/LDR, LDR/PCR, and PCR/RE/LDR experiments described in Projects 1 and 2 require synthesizing large numbers of oligonucleotides. The products from these cancer detection amplifications are currently separated and quantified on an ABI 373A DNA sequencer. Future detection will use oligonucleotide or PNA addressable arrays.

(ii) **Testing the efficiency and polymerase fidelity of nucleotide conversions using convertide oligonucleotides.** The PCR/RE/LDR cancer detection scheme has the potential of detecting cancer mutations at a sensitivity of 1 in  $10^6$  or  $10^7$ . The sensitivity of this scheme is dependent on the fidelity of polymerase extension off primers containing a 3' nucleotide analogue (Project 3). An assay has been developed to test both the efficiency and fidelity of different polymerases for each base conversion.

(iii) **Testing oligonucleotide or PNA addressable arrays for quantitative cancer mutation detection.** Large scale detection of a multitude of mutations will require addressable arrays. Mutations are distinguished by the position of a signal on the array. We plan to test arrays synthesized in Project 5 for fluorescent detection of LDR and LCR oligonucleotide products using a Molecular Dynamics FluorImager 575.

**B. FACILITIES OF THE INSTRUMENTATION AND MUTATION DETECTION CORE.**

The P.I.'s laboratory has an ABI 394 DNA synthesizer, an ABI 373A DNA sequencer, and a PE 9600 thermocycler which have been generously provided by industrial collaborators. This proposal requests the purchase of a Molecular Dynamics FluorImager 575, or equivalent fluorescent imager should another instrument become available in the first year. By the third year, many of these methods will be amenable to semi-automation, and we are requesting a fully programmable pipetting robot; either a Beckman Biomek or an ABI Catalyst 800. The purpose of the instrumentation core is to centralize mutation detection, and nucleotide analogue and array evaluation. The P.I. and several Co.-I's have sufficient interactions with industrial collaborators to have access to the next generation of instrumentation when it becomes available.

**C. EXPERIMENTAL METHODS AND DESIGN**

(i) **Provide instrumentation for oligonucleotide synthesis and analysis of cancer causing mutations.** The PCR/LDR, LDR/PCR, and PCR/RE/LDR experiments described in Projects 1 and 2 require synthesizing large numbers of oligonucleotides. The products from these cancer detection amplifications are currently separated and quantified on an ABI 373A DNA sequencer. Future detection will use oligonucleotide or PNA addressable arrays.

DNA diagnostics requires four steps which will all eventually be amenable to automation. These steps include (i) sample preparation, (ii) primer preparation, (iii) DNA amplification, and (iv) signal detection and analysis. Figs. 1 provides a flow chart for quantitative multiplex PCR/LDR detection of germline mutations. The principles may be illustrated for Li-Fraumeni syndrome patients who carry a mutation in the p53 gene. DNA prepared from such patients would be subjected to a multiplex PCR reaction that would amplify p53 exons 5 & 6, 7, and 8 & 9. The reaction mix would be diluted into a second tube which contains dozens of LDR primers. Addition of *Tth* ligase allows for allele specific linear amplification of the product, such that the ratio of the LDR products reflects their presence or absence in the two chromosomes. Two different types of LDR primers would be used, depending on the detection format. When detection is achieved in a linear dimension by gel or capillary electrophoresis, the LDR primers would be designed to give sequentially longer products. This can be accomplished by using poly A tails [1], or hexaethylene oxide tails [2, 3], to increase the molecular weight of the LDR product. The LDR primers are designed such that the wild-type and mutant alleles migrate adjacent to one another in the detection format. Presence of a germline mutation is easily distinguished by appearance of a new band on the electrophorogram. If the patient is homozygous for a particular mutation, then the original wild type signal disappears altogether, and only the mutant band is observed. This method of detecting multiple germline mutations has been successfully applied to cystic

fibrosis [2-4], hyperkalemic periodic paralysis [5], and 21 hydroxylase deficiency (D. Day, P. White, and F. Barany, unpublished, see Project 2).

To be able to assay for even more mutations, the LDR primers would be designed to contain unique zip codes which specifically hybridize to their complements on an addressable array. This allows for two dimensional detection. This method has the advantage of essentially uniform LDR primer length as well as higher throughput. In this format, the LDR primers are designed such that the wild-type and mutant alleles are captured adjacent to one another on the array. The amount of each allele is quantified by a Molecular Dynamics 575 Fluorometer. One convenient advantage of this format is its flexibility. An entirely new set of primers need not be resynthesized as additional mutations are discovered in between previously known mutations. The addressable array has the added advantage of being reusable.

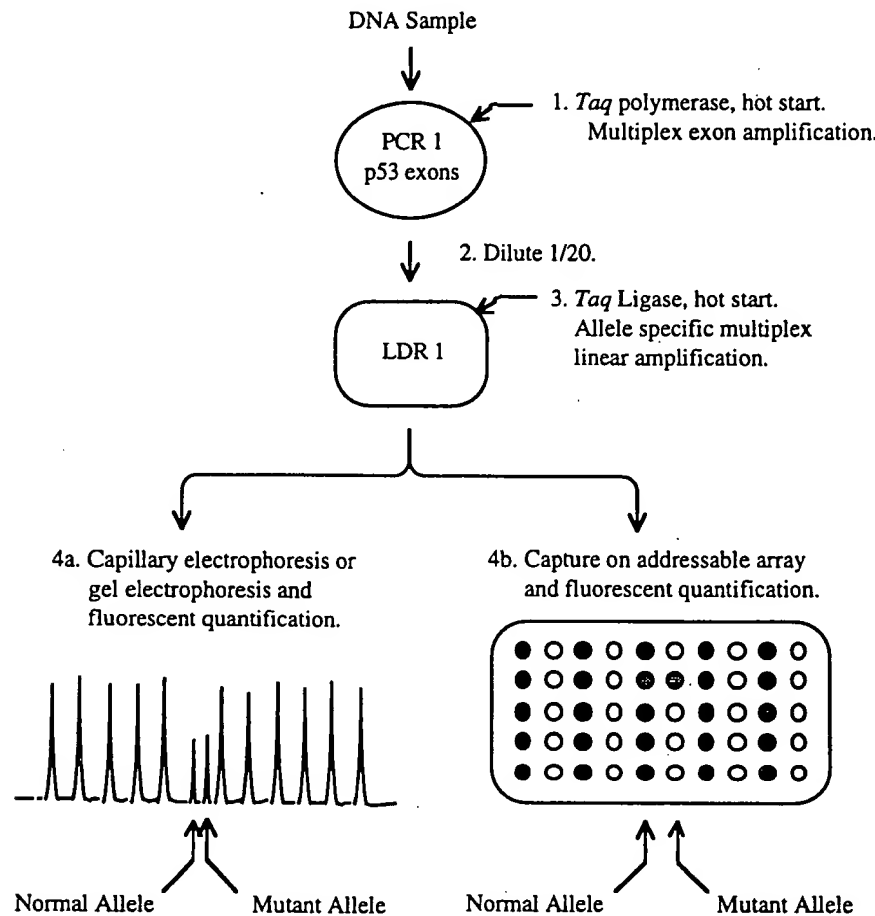


Fig. 1 PCR/LDR Flow chart for germline mutation detection. This diagram depicts detection of and germline point mutation, such as the p53 mutations responsible for Li-Fraumeni syndrome. 1. After DNA sample preparation, exons 5-8 are PCR amplified using *Taq* polymerase under hot start conditions. At end of the reaction, *Taq* polymerase is degraded by heating at 100°C for 10 min. 2. Products are diluted 20 fold into fresh LDR buffer containing allele specific and common LDR primers. A tube generally contains about 100 to 200 fmoles of each fragment. 3. The ligase reaction is initiated by addition of *Taq* ligase under hot start conditions. The LDR primers ligate to their adjacent primers only in the presence of target sequence which gives perfect complementarity at the junction site. The products may be detected in two different formats. 4a. In the first format, fluorescently labeled LDR primers contain different length poly A or hexaethylene oxide tails. Thus each product migrates with a slightly different mobility, giving a ladder of peaks. A germline mutation would generate a new peak on the electrophorogram. The size of the peaks will approximate the amount of the mutation present in the original sample; 0% for homozygous normal, 50% for heterozygous carrier, or 100% for homozygous mutant. 4b. In the second format, each allele-specific primer contains 24 additional nucleotides on their 5' ends. These sequences are unique "zip code" sequences which will specifically hybridize to their complementary zip code sequences on an addressable array. In the LDR reaction, each allele-specific primer can ligate to its adjacent fluorescently labeled common primer in the presence of the corresponding target sequence. Wild type and mutant alleles are captured on adjacent addresses on the array. Unreacted primers are washed away. The black dots indicate 100% signal for the

wild type allele. The white dots indicate 0% signal for the mutant alleles. The shaded dots indicate the one position of germline mutation, 50% signal for each allele.

Figs. 2 provides a flow chart for quantitative multiplex PCR/LDR detection of p53 cancer mutations. The aim is to quantify mutations which may be present in only a minority of the cells from a tissue sample. Thus, after PCR amplification, an aliquot is removed and analyzed by capillary electrophoresis or gel electrophoresis and each product band is quantified. The reaction is spiked with marker DNA, diluted in at 1/100 of the molar amount of the PCR product. This spiked marker will contain an artificially constructed mutation in a p53 template. Both cancer DNA and spiked DNA constitute a small percentage of the total DNA. However, PCR generates such large amounts of product, that 200 fmole of PCR product may be tested in an LDR reaction. If the mutant allele were only present at 1/200, then 199 fmole of product contains the wildtype sequence and only 1 fmole contains the mutant allele. For this detection, only a single mutant specific LDR primer is used. Ten rounds of LDR would generate somewhere between 5 and 10 fmole of product, depending on the efficiency of the LDR reaction. Typically, we load 10 to 20% of the LDR reaction on a sequencing gel. Thus loading 20% of product should give about 1 to 2 fmole of product in a given lane, which is well above the limit of detection (100 attomoles.) The wild type primer is not used in this form of

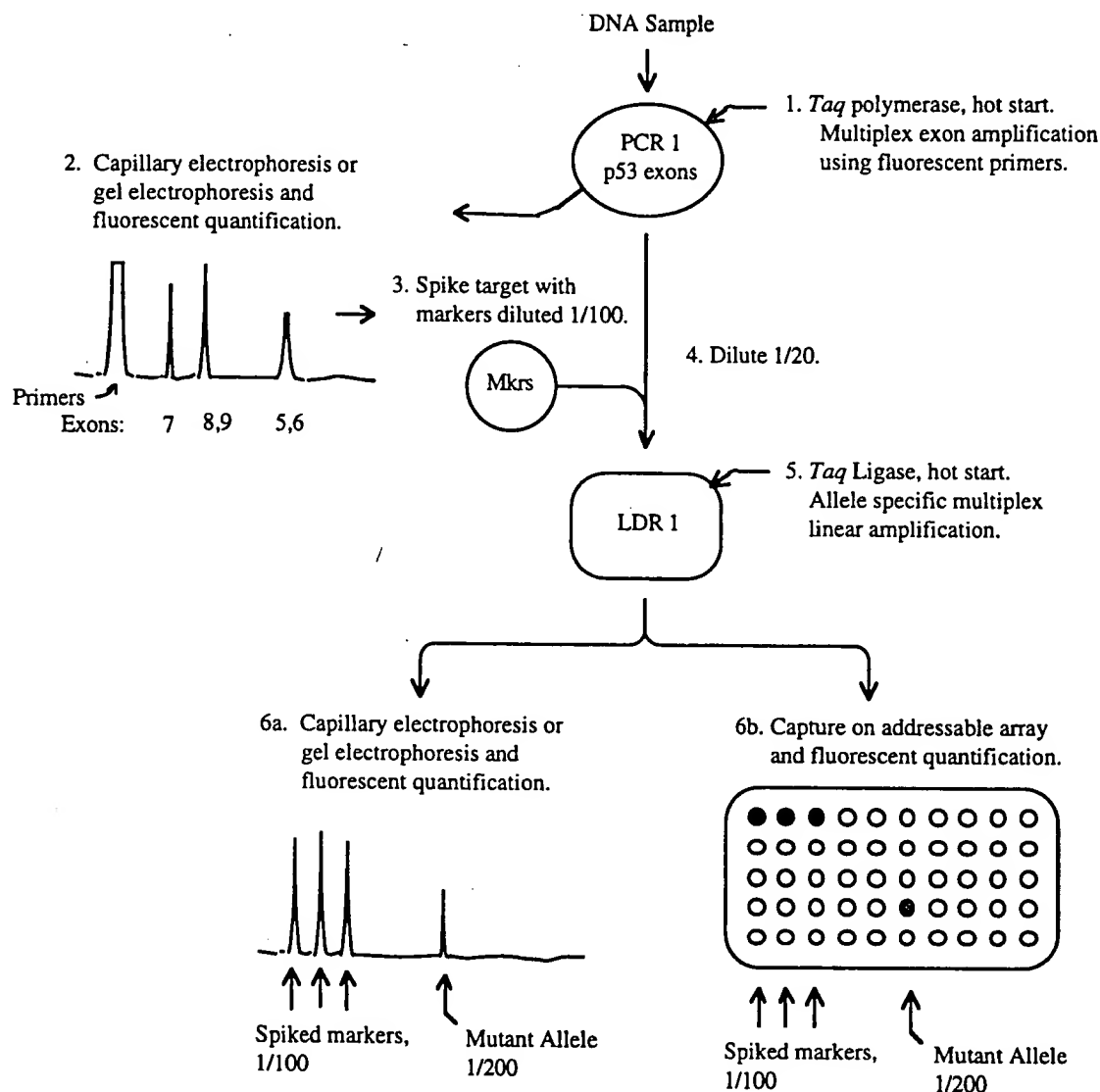


Fig. 2 PCR/LDR Flow chart of cancer mutation detection. This diagram depicts detection of somatic cell mutations in the p53 tumor suppressor gene, but is general for all low sensitivity mutation detection. 1. DNA samples are prepared and exons 5-9 are PCR amplified as three fragments using fluorescent PCR primers. 2. This allows for fluorescent quantification of PCR products using capillary or gel electrophoresis. 3. The products are spiked with a 1/100 dilution of marker DNA (for each of the three



fragments). This DNA is homologous to wild type DNA, except it contains a mutation which is not observed in cancer cells, but which may be readily detected with the appropriate LDR primers. 4. The mixed DNA products are diluted 20 fold into buffer containing all the LDR primers which are specific only to mutant or marker alleles. 5. The ligase reaction is initiated by addition of *Taq* ligase under hot start conditions. The LDR primers ligate to their adjacent primers only in the presence of target sequences which give perfect complementarity at the junction site. The products may be detected in the same two formats described in Fig. 1. 6a. Products are separated by capillary or gel electrophoresis and fluorescent signal are quantified. Ratios of mutant peaks to marker peaks give approximate amount of cancer mutations present in the original sample divided by 100. 6b. Products are detected by zip code specific hybridization to complementary sequences on an addressable array. Ratios of fluorescent signals in mutant dots to marker dots give approximate amount of cancer mutations present in the original sample divided by 100.

detection, since the wildtype signal would be so strong. The sensitivity of the detection could be increased even further by capturing the entire reaction mix on an addressable array. In Project 4 we have a major effort aimed at obtaining greater specificity in the ligase reaction. This could allow detection of one cancer cell mutation in  $10^3$  to  $10^4$  normal cells. At this level of sensitivity, it is possible that a standard LDR reaction might not generate sufficient product for detection of the cancer mutation. In such a case, one could switch to an LCR detection reaction, which has demonstrated sub-attomolar sensitivity [1]. For example, after 30 LCR cycles, the signal generated from 0.1 attomoles of the correct allele ( $B^A$ ) is eight fold higher than the signal from a 1,000 fold more of the incorrect allele ( $B^S$ ). LCR detection of low levels of a mutation is even compatible with multiplexing, since the discriminating base of every single primer will not base pair with wild type sequence present on target DNA nor on an adjacent overlapping complementary strand primer. In other words, if one uses dozens of LCR primers for detecting only mutant sequences, one need not be concerned about false positive signals from target independent ligation events.

(i) *Sample preparation.* The investigators in project 1 and 2 will be responsible for preparing DNA from frozen or fixed tissue, or bodily fluids. There have been two standard approaches to nucleic acid preparation; use of organic solvents such as phenol/chloroform, and use of silica based resins. The latter are available from several commercial vendors as gene or DNA purification kits. For example, Bioventures Inc., has a product termed Genereleaser, which is claimed to prepare PCR quality DNA from blood or tissue biopsies within 5 minutes. This and other DNA preparation methods will be compared to develop a standard procedure for DNA isolation. Some DNA samples from collaborators will also be sent as PCR amplified products. An ABI 341 Nucleic Acid Purification System instrument is commercially available for medium scale DNA preparation, but is currently limited to 8 samples. As advances are made in DNA detection, automated DNA preparation with high throughput should eventually become available.

(ii) *Primer preparation.* Full implementation of this program project grant will require the synthesis of a few hundred oligonucleotides over the course of the project. The P.I.'s laboratory has already synthesized over 100 oligonucleotides for projects 1 and 2 (see preliminary results), as well as for other LCR collaborators. The current ABI 392 DNA synthesizer has recently obtained an upgrade to a 4 column 8 bottle 394 model. This allows for simultaneous synthesis of oligonucleotides containing fluorescent as well as nucleotide analogues. Currently, oligonucleotide sequences are entered as Microsoft word text and copied directly into the OLIGONET program. In the future, this oligonucleotide design will be interfaced with the informatics core to allow for automated oligonucleotide synthesis. We routinely use polyacrylamide gel electrophoresis to obtain high purity oligonucleotides.

(iii) *DNA amplification.* The PE 9600 thermocycler is partially programmable, and has greatly aided the PCR/RE/LDR amplification experiments. Buffers, primers, enzymes, and formamide stop solutions are currently added manually, to allow for hot start amplifications. In the future, two types of improvements will speed up this process: (i) a robotic workstation, and (ii) silicon chip PCR reaction chambers.

A Beckman Biomek is a fully programmable pipetting robot with several stages for storing reagents, pipette tips. A thermocycler accessory is currently being developed and will be available by year three of the project. Alternatively, the ABI Catalyst 800 may be available in a fully programmable form which could achieve the same objectives. For the sake of illustration, consider the PCR/RE/LDR protocol. A PCR mixture could be made up by the robot and added to each of the samples on the thermal cycler. After each five thermal PCR cycles the reaction could be held at  $55^\circ\text{C}$  for five minutes to allow for addition of a thermophilic restriction enzyme which would selectively cleave normal but not mutant DNA. (Since the enzyme is not thermostable, it does not survive a PCR reaction, and needs to be added a few times. After an appropriate

number of cycles, an aliquot of the product could be removed. A new microtiter plate could be set up for thermal cycling with the LDR reagents. All of this could occur with no manual intervention other than adding the reagents to the appropriate wells at the beginning of the experiment and initiation of the program (to be provided by the informatics core). In the future, the Biomek will also come with a DNA preparation assembly which uses a silica based adsorption method to prepare high purity DNA from samples. This would allow the whole procedure from biological sample to labeled products to be performed without human intervention.

Ultimately, in collaboration with Dr. Allen Northrup of Lawrence Livermore Natl. Laboratory, and through our industrial collaborators Dr. Emily Winn-Deen of Applied Biosystems Division of Perkin Elmer, Dr. David Gelfand and Dr. John Sninsky of Roche Molecular Diagnostics, we plan to automate these reactions on silicon chips (See letters of collaboration). In preliminary designs, PCR amplification proceeded 4 times faster on chips than with the PE9600 thermocycler. The reaction chamber is etched in silicon, with an integrated polysilicon thin-film heater and feedback temperature control. Microfabrication technology will be used to further develop these devices to include serial PCR reactions ending with an LDR detection reaction. Such designs will automatically add reagents under hot start conditions, and dilute or electrophoretically move products into subsequent reaction or detection chambers. Since these devices will be self-contained, they will also solve PCR "carryover" problems.

An alternative strategy is being developed by Dr. Harold Swerdlow the University of Utah. PCR reactions are performed in a capillary electrophoresis tube, allow the entire reaction and product detection to proceed in a single instrument in about 30 min. This approach may also be amenable to automated PCR/LDR.

(iv) *Signal detection and analysis.* Current detection schemes are based on separation of LDR and LCR products by size on an automated ABI 373A DNA sequencer. Applying the Genescan 672 software allows for automatic quantification of each signal. An alternative approach separates DNA products using capillary electrophoresis [2, 3]. Both of these approaches are amenable to four color fluorescent labeling and detection. To dramatically expand the potential for multiplex mutation detection, addressable oligonucleotide or PNA arrays will be synthesized (see project 5.) LDR and LCR products will contain "zip code" sequences which will be captured at specific locations on the array which will contain "complementary zip code sequences." Fluorescent products will be detected and quantified by scanning the array with a Molecular Dynamics FluorImager 575. Data analysis will be aided by the informatics Core A.

(ii) **Testing the efficiency and polymerase fidelity of nucleotide conversions using convertide oligonucleotides.** The PCR/RE/LDR cancer detection scheme has the potential of detecting cancer mutations at a sensitivity of 1 in  $10^6$  or  $10^7$ . The sensitivity of this scheme is dependent on the fidelity of polymerase extension off primers containing a 3' nucleotide analogue (Project 3). An assay has been developed to test both the efficiency and fidelity of different polymerases for each base conversion.

(i) *A generalized assay to test the efficiency and polymerase fidelity of all 12 possible nucleotide conversions.* The nucleotide analogues generated in Project 3 will be incorporated into tester oligonucleotides to access their abilities to convert a given sequence into an endonuclease recognition site. We have devised a series of assays to determine how well a nucleotide analogue can "read" a natural base, and what bases the polymerase will "write" opposite the analogue (See Project 3, Scheme 2 for a more complete explanation).

The oligonucleotides in Fig. 3A. are designed to test the distribution of natural bases *Taq* polymerase incorporates ("writes") opposite a nucleotide analogue. Oligonucleotide QT 1Q3 will be synthesized with the "convertide" analogue in the middle, and subsequently PCR amplified using the two outside primers QT101 and QT102RB. The latter oligonucleotide primer is biotinylated on its 5' end, allowing for capture of the PCR products using streptavidin coated magnetic beads. The top strand is denatured off with base (beads washed), and resynthesized by adding 5' end labeled QT101 and extending with *Taq* polymerase. The resulting product contains perfectly double stranded DNA with an unknown distribution of natural bases at the position of the original analogue. Substitution of the analogue with a natural base creates a new restriction endonuclease recognition site for all four possibilities. By serially cutting with 4 restriction endonucleases, washing and counting the amount of product released after each digestion, the proportion of G, A, T, and C in the analogue position can be quantified. This assay will determine which bases the polymerase "writes" opposite the "convertide" and the proportion of each.

**A.**

QT 101	5'-	AT GTT GCT GAG CCC ACC T	-3'	18 mer
QT 1Q3	5'-	AT GTT GCT GAG CCC ACC TCG <u>QGC</u> ACG AGG CAG TAG TCT GAC	-3'	41 mer
QT 102RB	3'	TGC TCC GTC ATC AGA CTG B	-5'	18 mer

<i>TaqI</i>	TCG <u>A</u>
<i>XhoI</i>	C TCG <u>AG</u>
<i>AvaI</i>	C TCG <u>RG</u>
<i>BstI</i>	CCC ACC TCG <u>GG</u>
<i>BstUI</i>	CG <u>CG</u>
<i>HhaI</i>	G <u>CGC</u>
<i>MwoI</i>	G <u>CGC</u> ACG AGG C
<i>ApaLI</i>	G TGC AC
<i>DraIII</i>	C ACC TCG <u>TG</u>

**B.**

QT 104	5'-GCA TGT CCA GTC CAG GAT	-3'	18 mer
QT 105	5'-GCA TGT CCA GTC CAG GAT GTT GCT GAG CCC ACC TCG <u>A</u>	-3'	37 mer
QT 106	5'-GCA TGT CCA GTC CAG GAT GTT GCT GAG CCC ACC TCG <u>G</u>	-3'	37 mer
QT 107	5'-GCA TGT CCA GTC CAG GAT GTT GCT GAG CCC ACC TCG <u>C</u>	-3'	37 mer
QT 108	5'-GCA TGT CCA GTC CAG GAT GTT GCT GAG CCC ACC TCG <u>T</u>	-3'	37 mer
QT 2Q9	5' <u>CTA</u> GTT GCT GAG CCC ACC TCG <u>Q</u>	-3'	22 mer
QT 115R	3' <u>GAC</u> CAA CGA CTC GGG TGG AGC <u>AGC</u> TGC TCC GTC ATC AGA CTG	-5'	42 mer
QT 116R	3' <u>GAC</u> CAA CGA CTC GGG TGG AGC <u>GCG</u> TGC TCC GTC ATC AGA CTG	-5'	42 mer
QT 117R	3' <u>GAC</u> CAA CGA CTC GGG TGG AGC <u>CCG</u> TGC TCC GTC ATC AGA CTG	-5'	42 mer
QT 118R	3' <u>GAC</u> CAA CGA CTC GGG TGG AGC <u>TCG</u> TGC TCC GTC ATC AGA CTG	-5'	42 mer
QT 102RB	3' TGC TCC GTC ATC AGA CTG B	-5'	18 mer

**C.**

<i>Taq</i> MKR	3'	CTA CAA CGA CTC GGG TGG AGC <u>AGC</u> TGC TCC GTC ATC AGA CTG	-5'	42 mer
<i>BstI</i> MKR	3'	CTA CAA CGA CTC GGG TGG AGC <u>GCG</u> TGC TCC GTC ATC AGA CTG	-5'	42 mer
<i>Hae</i> MKR	3'	CTA CAA CGA CTC GGG TGG AGC <u>CCG</u> TGC TCC GTC ATC AGA CTG	-5'	42 mer
<i>Mbo</i> MKR	3'	CTA CAA CGA CTC GGG TGG AGC <u>TAG</u> TGC TCC GTC ATC AGA CTG	-5'	42 mer

Fig. 3. Oligonucleotides for testing the efficiency and polymerase fidelity of all 12 possible nucleotide analogue conversions. **A.** Sequences of three oligonucleotides designed to quantify the proportion of natural bases *Taq* polymerase inserts opposite a Q base nucleotide analogue. Insertion of: T generates a *TaqI* or *XhoI* site; C generates a *BstI* site; G generates a *BstUI*, *HhaI*, or *MwoI* site; and C generates an *ApaLI* or *DraIII* site. Oligonucleotide QT 1Q3 contains the nucleotide analogue in the middle, and is PCR amplified using primers QT 101 and QT 102RB. The latter oligonucleotide primer is biotinylated on its 5' end, allowing for capture of the PCR products using streptavidin coated magnetic beads. The top strand is removed by base, and labeled QT 101 reannealed for extension with *Taq* polymerase. Stepwise cleavage with the above restriction endonucleases, followed by counting the proportion of product released, will allow us to calculate the efficiency of incorporation of each base opposite a Q nucleotide analogue. **B.** Sequences of oligonucleotides designed to test the efficiency and fidelity of polymerase extension for every possible conversion. Oligonucleotides QT 115R-QT 118R represent the four possible target DNA sequences containing an "incorrect" base (A,G,C or T) adjacent to the "detection bases" (3' CG 5'). In the absence of "convertide" nucleotide analogues, one would hybridize one of the primers QT 105-QT 108 to target DNA, and with the bottom primer QT 102RB PCR amplify the target. These primers will convert the target sequence into a small duplex containing the restriction sites; *XhoI* generated by QT 105, *BstI* generated by QT 106, *HhaI* generated by QT 107, and *ApaLI* generated by QT 108. Since the reverse primer is biotinylated, products may be captured, treated with the above restriction enzyme, and then reamplified using outside primer QT 104 and QT 102RB. This is analogous to selective removal of normal DNA in the PCR/RE/LDR protocol. Reduced fidelity of *Taq* polymerase extension of the mismatch will be reflected by a higher percentage of endonuclease resistant product after two to three cycles of PCR/RE. The relative fidelity may be quantified by "spiking" the reaction mix with a million fold dilution of marker primers listed in part C. Each of these marker primers creates a *different* restriction site for each of the four bases in the position

flanking the test dinucleotide pair. At the end of two to three rounds of PCR/RE, the ratio of products released by the marker enzyme compared to unreleased product will reflect the relative fidelity of extension. Some of these conversions containing for example G:G mismatch will proceed at a very low efficiency if at all. Use of a nucleotide analogue primer QT 2Q9 in the first few rounds of the PCR cycle could significantly enhance both the efficiency and fidelity of such a conversion. Results using the nucleotide analogue will be compared to results using the natural bases as described above.

The oligonucleotides in Fig. 3 B & 3C are designed to test the efficiency and fidelity of polymerase extension for every possible conversion. The assay is designed to mimic the type of zip code conversion required for cancer detection. For illustrative purposes, we will describe the assay for testing the ability of analogue Q12 to convert a C into a T. The oligonucleotide QT 2Q9 will be synthesized with the nucleotide analogue Q12 on its 3'. This analogue containing oligonucleotide will be hybridized to oligonucleotide QT 116R, forming a Q12:G mismatch on the 3' end. The bottom strand primer, QT102RB is biotinylated for easy capture of product. Three rounds of PCR using the analogue primer will be followed by a dozen rounds of PCR using the longer zip code containing primer QT 108. The amount of product produced will be compared to that generated by a T:G mismatch (QT 108: QT 116R) or a T:A perfect match (QT 108: QT 115R). If polymerase extension of the nucleotide analogue is highly efficient, the amount of product will be equal to or nearly equal to polymerase extension of the perfect match.

To test the fidelity of extension, the product of the first PCR reaction is diluted, and reamplified using the zip code primer QT104, and the common biotinylated primer QT 102RB. Using the zip code primer ensures that the final amplification product does not contain shorter nucleotide analogue containing PCR products from the initial 3 rounds of nucleotide conversion amplification. A known amount of marker DNA, (the amplification product of QT 108: *Taq* MKR) is diluted into the zip code product at a concentration of one marker DNA/  $10^7$  test DNA. This marked zip code product will be subjected to endonuclease selection with *Apa*LI, and processed through a second round of nucleotide conversion, zip code amplification, and *Apa*LI selection. As described above, the top strand is denatured off, and resynthesized by extending 5' end labeled QT101. The resulting product contains perfectly double stranded DNA with an unknown distribution of the two bases *adjacent* to the position of the original analogue. By serially cutting with *Apa*LI and *Taq*I endonucleases, washing and counting the amount of product released after each digestion, the proportion of original DNA, marker DNA, and uncleavable product can be quantified. The uncleavable product represents incorrectly extended primer, and the ratio of uncleavable product to *Taq*I cleavable product gives the polymerase fidelity  $\times 10^{-7}$ . The results from this set of experiments will be compared with a control amplification starting with perfect match DNA (QT 108: QT 115R), to obtain the normal fidelity of *Taq* polymerase using synthetic templates.

This assay will determine the fidelity of extension for each nucleotide analogue mediated base conversion. If fidelity proves to be a problem with *Taq* polymerase, we will test higher fidelity proofreading polymerases using thiophosphate modified oligonucleotide substrates as described in Project 2. We aim to obtain conditions for converting any base into a restriction site with a fidelity of less than 1 error in  $10^7$  starting DNA molecules. This will allow us to completely generalize our PCR/RE/LDR method for detection of any cancer causing point mutation at sensitivity of 1 in  $10^6$  to 1 in  $10^7$  cells.

**(iii) Testing oligonucleotide or PNA addressable arrays for quantitative cancer mutation detection.** Large scale detection of a multitude of mutations will require addressable arrays. Mutations are distinguished by the position of a signal on the array. We plan to test arrays synthesized in Project 5 for fluorescent detection of LDR and LCR oligonucleotide products using a Molecular Dynamics FluorImager 575.

*(i) Initial test of 5 address zip codes.* A membrane or glass surface containing five different covalently attached spots of "complementary zip code" sequences will be used for our initial zip code tests. The PNA or oligonucleotide sequences will be spaced in a microtiter well format, allowing for easy washing steps after hybridization. Fluorescently labeled zip codes will be synthesized, and various quantities added to the surface of the membrane. Correct and mismatched sequences will be tested to establish signal to noise ratios. We plan to repeat the hybridization conditions developed for reverse dot blots by Zhang et al., (Zhang, 1991). Hybridization will be performed both with and without tetramethylammonium chloride (TMACl) to minimize  $T_m$  differences among the zip codes. (Use of TMACl will probably be unnecessary, especially if  $T_m$  values

of the zip codes have already been optimized by use of 5 propynyl U and primer design.) Washing membranes may be achieved more rapidly by using a dot blot apparatus. Glass surfaces will be washed by multiple changes of bulk liquid, as is done with standard Southern hybridizations. With PNA/DNA hybrids, we will also test washing in low salt to take advantage of the higher stability of PNA/DNA in these conditions.

Fluorescent signal will be detected by scanning the membrane or glass with a Molecular Dynamics FluorImager 575. Initial oligonucleotide or PNA spots will be quite large. These will help establish the limits of loading per surface area. By way of comparison, the current ABI 373A DNA sequencer can accurately detect about 100 attomoles of LDR product in a single band. A standard 20 $\mu$ l LDR reaction starts with 200 fmoles of labeled primer. One tenth of the reaction mix, or 20 fmoles is loaded per lane. Thus the signal to noise ratio is about 200 to 1. The Molecular Dynamics FluorImager is capable of detecting about 2 fmoles of signal per band in a gel. If the entire 20 $\mu$ l LDR mix containing 200 fmole of primer were allowed to hybridize on the same surface area, the signal to noise ratio would be about 100 to 1, assuming capture of all the LDR products during hybridization. However, this band covers 1000 pixels (1 pixel is about 50 $\mu$  X 50 $\mu$ ), so the true sensitivity could be as low as 2 attomoles per pixel. The important factor will be the amount of signal which may be captured in as small an area as possible. Although initial spots will cover large surface areas, we will try to saturate these spots to determine the loading per 50 $\mu$  pixel which is ultimately obtainable. The capacity will determine the ultimate signal to noise ratio achievable. For example, if 20 fmole of signal could be captured in a 200 $\mu$  square area, as little as 32 attomoles of signal could be detected, and a signal to noise ratio of 625 to 1 could be achieved.

(ii) *Testing the 25 address zip code array.* Once we have determined the optimal conditions for hybridizing zip codes to complementary zip codes, we can proceed with larger scale studies. A 25 address zip code array will be synthesized in Project 5. The remaining 20 fluorescently labeled zip code oligonucleotides will be synthesized as part of this core. All 25 zip code oligonucleotides will be added to the array to determine the melting temperatures of all the addresses. The membrane will be washed with hybridization buffer at increasingly higher temperatures, and the array scanned in the fluoroi-mager to determine the avidity of each zip code to its proper address. The experiment will be repeated using groups of 5 zip code oligonucleotides, each one occupying a unique row and column. The degree of cross hybridization to the incorrect addresses will be determined by repeating the melting experiments. Since PNA/DNA duplexes have higher T<sub>m</sub> values, we expect that PNA arrays will work in a higher and broader temperature range. This should allow for higher capture efficiencies and essentially no background hybridizations at incorrect addresses.

If these preliminary studies are successful, we will synthesize LDR primers with zip codes for detecting the 5 most common mutations in the p53 gene associated with breast cancer ( V157, R175, R248, R273 and R282, see project 2.) Results using the zip code capture method will be compared with our earlier results separating LDR products by size. Indeed, the new zip code containing ligation products will be designed to differ in size, so the same products may be detected using both methods and signal to noise ratios compared. When PCR/LDR detection of these five p53 codons is feasible, we will begin to apply the technique to our set of 100 to 200 primary tumor samples. Eventually we will expand the number of codons at which we can detect mutations to 24 using virtually all the addresses on the array. The last address will serve as the internal control for either wild-type sequence, or the marker sequence. DNA will be extracted from the specimens and simultaneous detection of mutations in these codons will be performed as described above. Finally, many of these tumors have been characterized for HER-2/neu amplification and the presence of bone marrow micrometastases by immunohistochemical staining. The types of mutations we find will be correlated with these laboratory parameters, the results of our studies of HER-2/neu and int-2 amplification in this tumor set (See project 2) and clinical prognostic parameters (e.g. primary tumor size, lymph node status and estrogen receptor status). Analogous studies could be performed with lung or colon cancer tissues.

**D. HUMAN SUBJECTS / VERTEBRATE ANIMALS:** Not applicable

**E. CONSULTANTS/COLLABORATORS:** Letters and Biographical Sketches for collaborators are attached in the overview section of this program project grant.

**F. CONSORTIUM/CONTRACTUAL ARRANGEMENTS:** None.

**G. LITERATURE CITED.**

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## **Core C.**

### **Administrative Core**

**Core Leader: Francis Barany  
Cornell University Medical College**

**Core Co-Leader: Michael Bunk  
Strang Cancer Prevention Center**

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. **DO NOT EXCEED THE SPACE PROVIDED.**

A summary of the administrative core structure, activities and responsibilities, and resources is presented in this section of the application.

PERSONNEL ENGAGED ON PROJECT, INCLUDING CONSULTANTS/COLLABORATORS. Use continuation pages as needed to provide the required information in the format shown below on *all* individuals participating in the project.

Name	<u>BARANY, Francis</u>	Degree(s)	<u>Ph.D.</u>	Social Security #	<u>REDACTED</u>
Position Title	<u>Associate Professor</u>	D.O.B.	<u>REDACTED</u>	Role on Project	<u>Prin. Investig.</u>
Organization	<u>Cornell Univeristy Medical College</u>			Department	<u>Microbiology</u>
Name	<u>BUNK, Michael</u>	Degree(s)	<u>Ph.D.</u>	Social Security #	<u>REDACTED</u>
Position Title	<u>Director of Research Management</u>	D.O.B.	<u>REDACTED</u>	Role on Project	<u>Co-investigator</u>
Organization	<u>Strang Cancer Prevention Center</u>			Department	<u>Administration</u>
Name		Degree(s)		Social Security #	
Position Title		D.O.B.		Role on Project	
Organization				Department	
Name		Degree(s)		Social Security #	
Position Title		D.O.B.		Role on Project	
Organization				Department	
Name		Degree(s)		Social Security #	
Position Title		D.O.B.		Role on Project	
Organization				Department	
Name		Degree(s)		Social Security #	
Position Title		D.O.B.		Role on Project	
Organization				Department	



DD

Principal Investigator/Program Director (Last, first, middle):  
**DETAILED BUDGET FOR INITIAL BUDGET PERIOD**  
**DIRECT COSTS ONLY**

F. BARANY, Ph.D.

FROM

94/12/01

THROUGH

95/11/30

PERSONNEL (Applicant Organization Only)				DOLLAR AMOUNT REQUESTED (omit cents)			
NAME	ROLE ON PROJECT	TYPE APPT. (months)	% EFFORT ON PROJ.	INST. BASE SALARY	SALARY REQUESTED	FRINGE BENEFITS	TOTALS
Francis Barany	Principal Investigator	12	5				
Michael Bunk	Admin. Core Co-Admin.	12	5				
To be appointed	Core	12	30				
<b>CORE C</b>							
<b>SUBTOTALS</b>					<b>\$17,300</b>	<b>\$5,536</b>	<b>\$22,836</b>
<b>CONSULTANT COSTS</b>							
Outside collaborators \$1,000							\$1,000
<b>EQUIPMENT (Itemize)</b>							
							\$0
<b>SUPPLIES (Itemize by category)</b>							
							\$0
<b>TRAVEL</b>							
Program scientific meeting \$9,000							\$9,000
<b>PATIENT CARE COSTS</b>		<b>INPATIENT</b>					\$0
		<b>OUTPATIENT</b>					\$0
<b>ALTERATIONS AND RENOVATIONS (Itemize by category)</b>							\$0
<b>OTHER EXPENSES (Itemize by category)</b>							
Phone, Xerox, Fax, Mail \$5,500							\$5,500
<b>SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD</b>							<b>\$38,336</b>
<b>CONSORTIUM/CONTRACTUAL COSTS</b>							
<b>DIRECT COSTS</b>				\$0	<b>TOTAL</b>		\$0
<b>INDIRECT COSTS</b>				\$0			
<b>TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD</b>							<b>\$38,336</b>

(Item 7a, Face Page)

**BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD  
DIRECT COSTS ONLY**

CORE C

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED			
			2nd	3rd	4th	5th
<b>PERSONNEL:</b>						
<i>Salary &amp; fringe benefits</i>						
<i>Applicant organization only</i>		\$22,836	\$45,584	\$47,407	\$49,303	\$51,275
<b>CONSULTANT COSTS</b>		\$1,000	\$1,040	\$1,082	\$1,125	\$1,170
<b>EQUIPMENT</b>		\$0	\$0	\$0	\$0	\$0
<b>SUPPLIES</b>		\$0	\$0	\$0	\$0	\$0
<b>TRAVEL</b>		\$9,000	\$9,360	\$9,734	\$10,123	\$10,528
<b>PATIENT CARE COSTS</b>	<b>INPATIENT</b>	\$0	\$0	\$0	\$0	\$0
	<b>OUTPATIENT</b>	\$0	\$0	\$0	\$0	\$0
<b>ALTERATIONS AND RENOVATIONS</b>		\$0	\$0	\$0	\$0	\$0
<b>OTHER EXPENSES</b>		\$5,500	\$5,720	\$5,949	\$6,187	\$6,434
<b>SUBTOTAL DIRECT COSTS</b>		\$38,336	\$61,704	\$64,172	\$66,738	\$69,407
<b>CONSORTIUM/ CONTRACTUAL COSTS</b>		\$0	\$0	\$0	\$0	\$0
<b>TOTAL DIRECT COSTS</b>		\$38,336	\$61,704	\$64,172	\$66,738	\$69,407
<b>TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD</b> (Item 8a)->						<b>\$300,357</b>

## JUSTIFICATION (Use continuation pages if necessary):

**From Budget for Initial Period:** Describe the specific functions of the personnel, collaborators, and consultants and identify individuals with appointments that are less than full time for a specific period of the year, including VA appointments.

**For All Years:** Explain and justify purchase of major equipment, unusual supplies requests, patient care costs, alterations and renovations, tuition remission, and donor/volunteer costs.

**From Budget for Entire Period:** Identify with an asterisk (\*) on this page and justify any significant increase or decrease in any category over the initial budget period. Describe any change in effort of personnel.

**For Competing Continuation Applications:** Justify any significant increases or decreases in any category over the current level of support.

**Personnel:** Cornell University Medical College and Strang Cancer Prevention Center salaries are in accordance with the high cost of living in New York City, as well as the experience of the personnel. A 5% effort and salary are requested by the Principal Investigator for the program project application. The P.I. will have overall responsibility for all projects and core areas. Cornell University Medical College has granted the Principal Investigator a Hirschl/Monique Weill-Caulier Career Scientist Award from 01/01/92 to 01/01/1997. This award of \$20,000 / year may be used as salary (and fringe benefit support) only. It thus allows the P.I. to spend full effort on research.

Dr. Micahel Bunk will provide detailed support with regard to all administrative aspects of this administrative core. He will be involved in administrating the collaborative aspects of the grant with other institutions and will directly supervise the grants coordination. Dr. Bunk will spend 5% effort in year 01 for

which no salary will be requested. Dr. Bunk will spend 10% effort in years 02 - 05 and we are requesting 10% salary in those years.

We are requesting a 30% salary for a research program coordinator for the first year. The research program coordinator will provide overall support to Drs. Barany and Bunk and other project investigators. He/she will have overall responsibility for day to day administrative support duties and will be the contact person for administrative personnel associated with other program laboratories contact person for administrative and accounting staff associated with each of the participating projects. He/she will also obtain financial reports from each collaborating research project. As his/her responsibilities will dramatically increase by the second year, we are requesting 50% salary for years 02-05. This will include coordinating an annual Program meeting.

*Fringe benefits:* Cornell University Medical College fringe benefits from 12/1/94 to 11/30/95 are at 32%. Salary increases of 4% are in accordance with Cornell University Medical College guidelines. Cornell University Medical College documentation of calculation of the indirect cost and fringe rate are attached.

*Consultant Costs:* We request a small amount to be paid as an honorarium for two outside speakers to our annual program project meeting. The speakers will critique our work.

*Travel::* We request support for travel for all Co-Investigators to an Annual Program Meeting. The meeting will alternate between Cornell University Medical College/Strang Cancer Prevention Center in New York City and the National Cancer Institute in Bethesda to present progress on the cancer detection work. We also request support for travel of two outside speakers /consultants to this annual meeting.

*Other expenses.* We request support for phone, Fax, printing, and photocopying of \$5,500 for the first year. This also includes consumable supplies such as pens, computer disks, other computer supplies, fax paper, printer cartridges, and other miscellaneous program supplies for the administrative core.

## SUMMARY OF THE ADMINISTRATIVE CORE

### INTRODUCTION

The structure of the administrative core has been designed to meet the planning and managerial needs of the program project. It is also aimed at combining the expertise of all institutional administrators and scientific program directors in order to enhance the proficiency of the research level in order to promote cooperative efforts at all collaborating institutions.

The administrative responsibility of the Administrative core will include: 1) monitoring the scientific and administrative diligence of each of the component projects and re-allocating resources when and if necessary, 2) assuring that the network of communication and collaboration is developed and maintained, 3) scheduling meetings of group investigators to be held approximately three times a year, 4) to keep the NIH Scientific Program Director/Coordinator apprised of group progress, changes in scientific aims, personnel, etc. 5) preparing progress reports for the NIH 6) assuring that core resources are providing adequate support to all projects, 7) coordinating group activities with all external companies in keeping with the formal collaborative research arrangements, and 8) monitoring inventions and invention disclosures supported by the program. In this fashion, the Administrative core will enhance maximum coordination between investigators participating in the program and facilitate the research objectives of the program project.

### ADMINISTRATIVE ACCOUNTABILITY

Staff members of the administrative core will be responsible to the Principal Investigator and Scientific Program Director, and will coordinate the daily interactions between investigators and collaborating institutional administrators.

**Dr. Francis Barany**, Principal Investigator, will have overall responsibility for the program project and will be Co-Director of the Administrative Core. The P.I. considers himself fortunate to be under the mentorship of Dr. Kenneth I. Berns, Chairman of the Department of Microbiology, whose administrative skills includes being Chairman of the Board of Public and Scientific Affairs of the American Society for Microbiology, Past President of the American Society of Virology, and Chairman elect of the Association of American Medical Colleges. In addition, Dr. Michael Selsted of the University of California, Irvine has been program director of a similar size application for the past 4 years. Dr. Selstead collaborates with Dr. G. Barany, and has provided invaluable advice on organization and administration of a program project.

**Dr. Michael Bunk** will serve as Co-Director of the Administrative Core with Dr. Barany. Dr. Bunk will be responsible for the overall research management of the program project including the preparation of institutional, scientific and financial reports as well as reports required by the National Institutes of Health and collaborating institutions. Dr. Bunk is also responsible, along with the financial administration department of the institute, for the proper filing of accounting forms, program accountability of expenditures and supervision of funds, maintenance of records and the administrative coordination between collaborating investigators and administrators. His duties also include initializing and monitoring an advisory committee for the program project, maintaining files and submitting all progress reports and renewal applications.

The Administrative Core Program Coordinator, **to be named**, will assist Drs. Barany and Bunk as well as collaborating project investigators. His/her capacity, as it relates to the administrative core, will be to manage daily activities of the program project, inter-relating projects, cores and other related programs. He/she will be involved and oversee all aspects of the project and will have considerable responsibility for collecting and maintaining research related information and data and maintaining telephone contact with all collaborating institutions. The project coordinator will have direct line responsibility with all research assistants and accountants at collaborating institutions.

## *ANNUAL PROGRAM MEETING*

This program project application represents an integrated and focused multi-disciplinary approach to cancer, genetic and infectious disease detection. Our interactive team (Principal and co-Investigators, academic and industrial collaborators) spans the areas of molecular biology, clinical oncology, medical genetics, microbiology, biological and organic chemistry, protein engineering, structural biology, microfabrication engineering, and information science. Due to the diversity of fields among both collaborators and co-investigators, it is important that there are annual meetings. These meetings will serve not only as a means of presenting progress among the individual labs, but also as a means of furthering our understanding of the molecular mechanics of cancers. With this in mind, we plan to invite two internationally renowned experts in cancer or genetic diseases to each meeting. These experts may be from our current collaborators, or outside. For the first year, we are considering inviting Dr. John Kovach from the Mayo Clinic, Dr. Steven Friend from Harvard Medical School, or Dr. Mark Sobel from the National Cancer Institute. We would also like to invite local experts, for example Dr. Larry Norton and Dr. Jerard Hurwitz from Memorial Sloan Kettering Cancer Institute for meetings in New York City, or Dr. Curtis Harris of the National Cancer Institute for meetings in Bethesda. Invited experts will evaluate our work and make critical suggestions for improving our research. In addition, the experts will speak about their own works in progress. An honorarium of \$500 is requested for each speaker for the first year. Organization of the program will be handled by the administrative core program coordinator.

## *CONSORTIUM/CONTRACTUAL AGREEMENTS*

As the applicant organization, Cornell University Medical College will administer the award through the Office of Sponsored Research at Cornell University Medical College. The portions of the award supporting activities at off-campus sites will be administered as subcontracts to those institutions. Consortium letters are appended on the next few pages. Subcontract awards will be made to Purdue University School of Pharmacy & Pharmacal Sciences, Louisiana State University, College of Physicians & Surgeons of Columbia University, and the University of Minnesota. Indirect costs from each of these institutions have been included in the overall direct cost of this project. Cornell University Medical College and the Strang Cancer Prevention Center are already linked through their joint affiliation with The New York Hospital. Administration of all subcontracts will be in compliance with Federal regulations and policies.

**CORNELL UNIVERSITY MEDICAL COLLEGE**  
**DEPARTMENT OF MICROBIOLOGY**

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1300 YORK AVENUE, Box 62  
NEW YORK, N.Y. 10021  
Telephone: (212) 746-6505  
Fax: (212) 746-8587

**STATEMENT OF INTENT TO ESTABLISH A CONSORTIUM AGREEMENT**

**Date:** January 26, 1994

**Grant Number:** P01-

**P-01 Application Title:** PROGRAM PROJECT: NEW METHODS FOR  
CANCER DETECTION

Project # 1; GENETIC MARKERS OF LUNG AND  
COLON CANCER.

**Proposed Project Period:** Year 01; 12/01/94- 11/30/95

"The appropriate programmatic and administrative personnel of each institution involved in this grant application are aware of the NIH consortium grant policy and will establish the necessary inter-institutional agreement(s) consistent with that policy."

**CORNELL UNIVERSITY MEDICAL  
COLLEGE, NEW YORK, NY**

(Applicant Institution)

**CHILDRENS HOSPITAL  
DENVER, CO.**

(Consortium Institution)

\_\_\_\_\_  
(name) (date)  
Principal Investigator:

**FRANCIS BARANY, Ph.D.**

\_\_\_\_\_  
(name) (date)  
Co-Investigator:

**VINCENT L. WILSON, Ph.D.**

\_\_\_\_\_  
(name) (date)  
Official Authorized to Sign for Institution

**GREGORY W. SISKIND, M.D.  
ASSOCIATE DEAN**

\_\_\_\_\_  
(name) (date)  
Official Authorized to Sign for Institution



1300 YORK AVENUE, Box 62  
NEW YORK, N.Y. 10021  
Telephone: (212) 746-6305  
Fax: (212) 746-8387

STATEMENT OF INTENT TO ESTABLISH A CONSORTIUM AGREEMENT

Date: January 26, 1994

Grant Number: P01-

P-01 Application Title: PROGRAM PROJECT: NEW METHODS FOR  
CANCER DETECTION

Project # 3; DESIGN AND SYNTHESIS OF  
NUCLEOTIDE ANALOGUES.

Proposed Project Period: Year 01; 12/01/94- 11/30/95

"The appropriate programmatic and administrative personnel of each institution involved in this grant application are aware of the NIH consortium grant policy and will establish the necessary inter-institutional agreement(s) consistent with that policy."

CORNELL UNIVERSITY MEDICAL  
COLLEGE, NEW YORK, NY

(Applicant Institution)

LOUISIANA STATE UNIV.  
BATON ROUGE, LA.

(Consortium Institution)

\_\_\_\_\_  
(name) (date)  
Principal Investigator:

FRANCIS BARANY, Ph.D.

\_\_\_\_\_  
(name) (date)  
Co-Investigator:

ROBERT HAMMER, Ph.D.

\_\_\_\_\_  
(name) (date)  
Official Authorized to Sign for Institution

GREGORY W. SISKIND, M.D.  
ASSOCIATE DEAN

\_\_\_\_\_  
(name) (date)  
Official Authorized to Sign for Institution

**CORNELL UNIVERSITY MEDICAL COLLEGE**  
**DEPARTMENT OF MICROBIOLOGY**



1300 YORK AVENUE, Box 62  
 NEW YORK, N.Y. 10021  
 Telephone: (212) 746-6505  
 Fax: (212) 746-8587

**STATEMENT OF INTENT TO ESTABLISH A CONSORTIUM AGREEMENT**

**Date:** January 26, 1994

**Grant Number:** P01-

**P-01 Application Title:** PROGRAM PROJECT: NEW METHODS FOR  
 CANCER DETECTION

Project # 3; DESIGN AND SYNTHESIS OF  
 NUCLEOTIDE ANALOGUES.

**Proposed Project Period:** Year 01; 12/01/94- 11/30/95

"The appropriate programmatic and administrative personnel of each institution involved in this grant application are aware of the NIH consortium grant policy and will establish the necessary inter-institutional agreement(s) consistent with that policy."

**CORNELL UNIVERSITY MEDICAL  
 COLLEGE, NEW YORK, NY**

(Applicant Institution)

**PURDUE UNIVERSITY  
 WEST LAFAYETTE, IN.**

(Consortium Institution)

\_\_\_\_\_  
 (name) (date)  
 Principal Investigator:

**FRANCIS BARANY, Ph.D.**

\_\_\_\_\_  
 (name) (date)  
 Co-Investigator:

**DONALD BERGSTROM, Ph.D.**

\_\_\_\_\_  
 (name) (date)  
 Official Authorized to Sign for Institution

**GREGORY W. SISKIND, M.D.  
 ASSOCIATE DEAN**

\_\_\_\_\_  
 (name) (date)  
 Official Authorized to Sign for Institution



**CORNELL UNIVERSITY MEDICAL COLLEGE**  
**DEPARTMENT OF MICROBIOLOGY**



1300 YORK AVENUE, Box 62  
NEW YORK, N.Y. 10021  
Telephone: (212) 746-6505  
Fax: (212) 746-8587

**STATEMENT OF INTENT TO ESTABLISH A CONSORTIUM AGREEMENT**

**Date:** January 26, 1994

**Grant Number:** P01-

**P-01 Application Title:** PROGRAM PROJECT: NEW METHODS FOR  
CANCER DETECTION

Project # 4; ENGINEERING AN IMPROVED  
THERMOSTABLE LIGASE.

**Proposed Project Period:** Year 01; 12/01/94- 11/30/95

"The appropriate programmatic and administrative personnel of each institution involved in this grant application are aware of the NIH consortium grant policy and will establish the necessary inter-institutional agreement(s) consistent with that policy."

**CORNELL UNIVERSITY MEDICAL  
COLLEGE, NEW YORK, NY**

(Applicant Institution)

**COLUMBIA UNIVERSITY  
NEW YORK, NY.**

(Consortium Institution)

\_\_\_\_\_  
(name) (date)  
**Principal Investigator:**

**FRANCIS BARANY, Ph.D.**

\_\_\_\_\_  
(name) (date)  
**Co-Investigator:**

**ANEEL AGGARWAL, Ph.D.**

\_\_\_\_\_  
(name) (date)  
**Official Authorized to Sign for Institution**

**GREGORY W. SISKIND, M.D.**  
**ASSOCIATE DEAN**

\_\_\_\_\_  
(name) (date)  
**Official Authorized to Sign for Institution**

**CORNELL UNIVERSITY MEDICAL COLLEGE**  
**DEPARTMENT OF MICROBIOLOGY**



1300 YORK AVENUE, Box 62  
NEW YORK, N. Y. 10021  
Telephone: (212) 746-6305  
Fax: (212) 746-8587

**STATEMENT OF INTENT TO ESTABLISH A CONSORTIUM AGREEMENT**

**Date:** January 26, 1994

**Grant Number:** P01-

**P-01 Application Title:** PROGRAM PROJECT: NEW METHODS FOR  
CANCER DETECTION

**Project # 5; DESIGN AND SYNTHESIS OF DNA AND  
PNA ARRAYS.**

**Proposed Project Period:** Year 01; 12/01/94- 11/30/95

"The appropriate programmatic and administrative personnel of each institution involved in this grant application are aware of the NIH consortium grant policy and will establish the necessary inter-institutional agreement(s) consistent with that policy."

**CORNELL UNIVERSITY MEDICAL  
COLLEGE, NEW YORK, NY**

(Applicant Institution)

**UNIVERSITY OF MINNESOTA  
MINNEAPOLIS, MN.**

(Consortium Institution)

\_\_\_\_\_  
(name) (date)  
**Principal Investigator:**

**FRANCIS BARANY, Ph.D.**

\_\_\_\_\_  
(name) (date)  
**Co-Investigator:**

**GEORGE BARANY, Ph.D.**

\_\_\_\_\_  
(name) (date)  
**Official Authorized to Sign for Institution**

**GREGORY W. SISKIND, M.D.  
ASSOCIATE DEAN**

\_\_\_\_\_  
(name) (date)  
**Official Authorized to Sign for Institution**

CORNELL UNIVERSITY MEDICAL COLLEGE  
DEPARTMENT OF MICROBIOLOGY



1300 YORK AVENUE, Box 62  
NEW YORK, N.Y. 10021  
Telephone: (212) 746-6505  
Fax: (212) 746-8587

STATEMENT OF INTENT TO ESTABLISH A CONSORTIUM AGREEMENT

**Date:** January 27, 1994

**Grant Number:** P01-

**P-01 Application Title:** PROGRAM PROJECT: NEW METHODS FOR  
CANCER DETECTION

CORE C: ADMINISTRATIVE CORE.

**Proposed Project Period:** Year 01; 12/01/94- 11/30/95

"The appropriate programmatic and administrative personnel of each institution involved in this grant application are aware of the NIH consortium grant policy and will establish the necessary inter-institutional agreement(s) consistent with that policy."

CORNELL UNIVERSITY MEDICAL  
COLLEGE, NEW YORK, NY

(Applicant Institution)

STRANG CANCER PREVENTION CTR.  
NEW YORK, NY

(Consortium Institution)

\_\_\_\_\_  
(name) (date)  
Principal Investigator:

FRANCIS BARANY, Ph.D.

\_\_\_\_\_  
(name) (date)  
Co-Investigator:

MICHAEL J. BUNK, Ph.D.

\_\_\_\_\_  
(name) (date)  
Official Authorized to Sign for Institution

GREGORY W. SISKIND, M.D.  
ASSOCIATE DEAN

\_\_\_\_\_  
(name) (date)  
Official Authorized to Sign for Institution

## TABLE OF CORE USAGE

	Project 1	Project 2	Project 3	Project 4	Project 5
Core A Informatics	30%	30%	10%	-	10%
Core B Instrumentation	20%	25%	25%	10%	20%
Core C Administration	20%	20%	20%	20%	20%

## CHECKLIST

## TYPE OF APPLICATION

- ☒ NEW application. (This application is being submitted to the PHS for the first time.)
- ☐ REVISION of application number: \_\_\_\_\_  
(This application replaces a prior unfunded version of a new, competing continuation, or supplemental application.)
- ☐ COMPETING CONTINUATION of grant number: \_\_\_\_\_  
(This application is to extend a funded grant beyond its current project period.)
- ☐ SUPPLEMENT to grant number: \_\_\_\_\_  
(This application is for additional funds to supplement a currently funded grant.)
- ☐ CHANGE of principal investigator/program director.  
Name of former principal investigator/program director: \_\_\_\_\_
- ☐ FOREIGN application, city and country of birth and present citizenship of principal investigator/program director. (This information is required by the U.S. Department of State.) \_\_\_\_\_

## 1. ASSURANCES/CERTIFICATIONS

The following assurances/certifications are made by checking the appropriate boxes and are verified by the signature of the OFFICIAL SIGNING FOR APPLICANT ORGANIZATION on the FACE PAGE of the application. Descriptions of individual assurances/certifications begin on page 24 of Specific Instructions.

- a. Human Subjects (Complete Item 4 on the Face Page)  
☐ Full IRB Review ☐ Expedited Review
- b. Vertebrate Animals (Complete Item 5 on the Face Page)
- c. Inventions and Patents (Competing Continuation Application Only—Complete Item 10 on the Face Page)
- d. Debarment and Suspension ☒ No ☐ Yes (Attach explanation)
- e. Drug-Free Workplace (Applicable only to new or revised applications being submitted to the PHS for the first proposed project period, Type 1)  
☒ Yes ☐ No (Attach explanation)
- f. Lobbying  
With Federal appropriated funds ☒ No  
With other than Federal appropriated funds ☒ No ☐ Yes  
(If "Yes," see page 29 and attach Standard Form LLL, "Disclosure of Lobbying Activities," to the application behind the second page of the Checklist.)
- g. Delinquent Federal Debt ☒ No ☐ Yes (Attach explanation)
- h. Misconduct in Science (Form PHS 6315) ☒ Filed ☐ Not Filed  
If filed, date of Initial Assurance or latest Annual Report 2/4/93
- |   |  |   |   |
|---|--|---|---|
| i. Civil Rights<br>Form HHS 441           | j. Handicapped Individuals<br>Form HHS 641 | k. Sex Discrimination<br>Form HHS 639-A   | l. Age Discrimination<br>Form HHS 680     |
| <input checked="" type="checkbox"/> Filed | <input checked="" type="checkbox"/> Filed  | <input checked="" type="checkbox"/> Filed | <input checked="" type="checkbox"/> Filed |
| <input type="checkbox"/> Not Filed        | <input type="checkbox"/> Not Filed         | <input type="checkbox"/> Not Filed        | <input type="checkbox"/> Not Filed        |

**CHECKLIST (Continued)****2. PROGRAM INCOME** (See instructions, page 32.)

All applications must indicate (Yes or No) whether program income is anticipated during the period(s) for which grant support is requested.

☒ No ☐ Yes If "Yes," use the format below to reflect the amount and source(s) of anticipated program income.

Budget Period	Anticipated Amount	Source(s)

**3. INDIRECT COSTS**

Indicate the applicant organization's most recent indirect cost rate established with the appropriate DHHS Regional Office, or, in the case of forprofit organizations, the rate established with the appropriate PHS Agency Cost Advisory Office. If the applicant organization is in the process of initially developing or renegotiating a rate, or has established a rate with another Federal agency, it should, immediately upon notification that an award will be made, develop a tentative indirect cost rate proposal. This is to be based on its most recently completed fiscal year in accordance with the principles set forth in the pertinent *DHHS Guide for Establishing Indirect Cost Rates*, and submitted to the appropriate DHHS Regional Office or PHS Agency Cost Advisory Office. Indirect costs will **not** be paid on foreign grants, construction grants, grants to Federal organizations, and grants to individuals, and usually not on conference grants. Follow any additional instructions provided for Research Career Development Awards, Institutional National Research Service Awards, and the specialized grant applications listed on page 6.

☒ DHHS Agreement dated: 5/28/93 ☐ No Indirect Costs Requested.☐ DHHS Agreement being negotiated with \_\_\_\_\_ Regional Office.☐ No DHHS Agreement, but rate established with \_\_\_\_\_ Date \_\_\_\_\_**ALCULATION\***

(The entire grant application, including the Checklist, will be reproduced and provided to peer reviewers as CONFIDENTIAL information. Supplying the following information on indirect costs is OPTIONAL for forprofit organizations.)

**a. Initial budget period:**Amount of base \$ 361,569 x Rate applied 70.2 % = Indirect costs (1) \$ 253,821**b. Entire proposed project period:**Amount of base \$ 2,420,832 x Rate applied 70.2 % = Indirect costs (2) \$ 1,699,424

(1) Add to total direct costs from form page 4 and enter new total on FACE PAGE, Item 7b.

(2) Add to total direct costs from form page 5 and enter new total on FACE PAGE, Item 8b.

\*Check appropriate box(es):

☐ Salary and wages base ☒ Modified total direct costs base ☐ Other base (Explain below)☐ Off-site, other special rate, or more than one rate involved (Explain below)

Explanation (Attach separate sheet, if necessary.):



DEPARTMENT OF THE NAVY  
OFFICE OF NAVAL RESEARCH  
RESIDENT REPRESENTATIVE  
33 THIRD AVENUE, LOWER LEVEL  
NEW YORK, NEW YORK 10003-9998

IN REPLY REFER TO

28 MAY 93

NEGOTIATION AGREEMENT

INSTITUTION: Cornell University Medical College  
1300 York Avenue  
New York, N.Y. 10021

## SECTION I: RATES

The indirect cost rates contained herein are for use on grants and contracts with all Federal agencies in accordance with the cost principles mandated by the Office of Management and Budget (OMB) Circular A-21, and subject to the conditions contained in Section II. These rates shall be used for forward-pricing and billing purposes until amended.

## A. FIXED INDIRECT COST RATES (WITH CARRY-FORWARD PROVISIONS)

Type	From	To	Rate	Base	Applicable to	Location
Fixed	7/1/93	6/30/94	70.20%	(a)	Research (Including Res. Training)	On-Campus
Fixed	7/1/93	6/30/94	38.65%	(a)	Research (Including Res. Training)	Off-Campus Westchester
Fixed	7/1/93	6/30/94	42.50%	(b)	Research	Clinical Res. Ctr.
Fixed	7/1/93	6/30/94	28.70%	(a)	Research (Including Res. Training)	Off-Campus

Base: (a) Modified Total Costs Excluding:

- (1) Permanent equipment costs
- (2) Alteration and renovation costs
- (3) Patient care cost
- (4) Tuition (Research Training)
- (5) Costs in excess of \$25,000 of each subcontract/grant

INSTITUTION: CORNELL UNIVERSITY MEDICAL COLLEGE

3. The off-campus rates are established for research and research training performed for 90 days or more at the Westchester Division of New York Hospital located in White Plains, N.Y. and all other off-campus locals.

4. Carry forward adjustments stated on the components of the published indirect cost rate for FY 1994 shall apply.

BY THE INSTITUTION

CORNELL UNIV. MEDICAL COLLEGE  
(Institution)

*Philip V. Giuca*  
(Signature)

PHILIP V. GIUCA  
(Name)

Senior Associate Dean - Finance  
(Title)

28 MAY 1993  
(Date)

BY THE COGNIZANT AGENCY  
ON BEHALF OF THE FEDERAL  
GOVERNMENT

OFFICE OF NAVAL RESEARCH  
(Agency)

*Gustav Bellisari*  
(Signature)

GUSTAV BELLISARI  
(Name)

Contracting Officer  
(Title)

28 MAY 1993  
(Date)

Telephone: (212) 529-3673



INSTITUTION: CORNELL UNIVERSITY MEDICAL COLLEGE

- C. FIXED RATES: If a fixed rate is in the Agreement, it is based on an estimate of the costs for the period covered by the rate. When the actual costs for this period are determined, an adjustment will be made to a rate of a future year(s) to compensate for the difference between the costs used to establish the fixed rate and actual costs.
- D. USE BY OTHER FEDERAL AGENCIES: The rates in this Agreement were approved in accordance with the authority of Office of Management and Budget Circular A-88, and should be applied to grants, contracts, and other agreements covered by Office of Management and Budget Circular A-21, subject to any limitations in A. above. The institution may provide copies of the agreement to other Federal Agencies to give them early notification of the Agreement.
- E. SPECIAL REMARKS:
1. Purpose of this agreement is to establish fixed overhead rates with carry-forward provisions for the period 01 July 1993 to 30 June 1994. Rates stated in section I above are based on the institution's proposal dated 26Feb93 supplemented by correspondence of 29Apr93 and 12May93, and DCAA audit report 2211-92J23000345 of 7May93.
  2. The fixed rates contained in this agreement are based on the institution's cost allocation special studies for Library Utilization; Weighted Utilities; Student Administration; sponsored Projects; Animal Center, and Space Utilization Survey. Establishment of these Fixed rates does not constitute Government acceptance of these special studies for FY 1994 or any prior fiscal year. The Government has not had adequate time to fully review these studies. Therefore, the institution's final indirect cost rates will be subject to increase or decrease, depending upon the negotiated outcome of the Government's review.

INSTITUTION;     CORNELL UNIVERSITY MEDICAL COLLEGE

- (b) Modified Total Direct Costs, excluding
  - (1) Permanent equipment costs
  - (2) Alteration and renovation cost
  - (3) Patient care cost
  - (4) Nurses, dieticians, social workers,  
New York Hospital
  - (5) Cost in excess of \$25,000 of each  
subcontract/grant

SECTION II:     GENERAL

A. LIMITATIONS:     The rates in this Agreement are subject to any statutory or administrative limitations and apply to a given grant, contract or other agreement only to the extent that the funds are available. Acceptance of the rates is subject to the following conditions: (1) Only costs incurred by the institution were included in its indirect cost pool as finally accepted; such costs are legal obligations of the institution and are allowable under the governing cost principles; (2) The same costs that have been treated as indirect costs are not claimed as direct costs; (3) Similar types of costs have been accorded consistent accounting treatment, and (4) The information provided by the institution which was used to establish the rates is not later found to be materially incomplete or inaccurate.

B. ACCOUNTING CHANGES:     If a fixed or predetermined rate is in this Agreement, it is based on the accounting system purported by the institution to be in effect during the Agreement period. Changes to the method of accounting for costs which affect the amount of reimbursement resulting from the use of this agreement require prior approval of the authorized representative of the cognizant agency. Such changes include, but are not limited to changes in the charging of a particular type of cost from indirect to direct. Failure to obtain approval may result in cost disallowances.

**Appendix 3**  
(To Declaration of Gerald Zon under 37 CFR § 1.608(b))



# DRAFT REVIEW REPORT

FRANCIS BARANY, PH.D.

1 PO1 CA65930-01

"NEW METHODS FOR CANCER DETECTION"

CORNELL UNIVERSITY MEDICAL COLLEGE  
NEW YORK, NEW YORK

Committee/Date: Special Review Subcommittee C/July 20-22, 1994

Review Meeting/Date Held: Site Visit/May 31-June 2, 1994

Special Notes: None

## OUTSIDE OPINION OBTAINED

Administrative Note: Page 19.

Resume: Funds are requested for this application that describes a series of exciting technological advances for detecting high sensitivity mutations that could have a profound impact on the diagnosis, and eventually therapy, of human cancer. The application describes several clinical situations where high resolution detection of mutations in general, and base substitution mutations in particular, could have a major impact on the early detection and diagnosis of cancer, prognostication based on micrometastases at the time of diagnosis, and the early detection of recurrence. The strengths include the potential of the proposed studies, and the high capability of the investigators to carrying out the proposed studies. The weaknesses include the overly ambitious nature of the application, a lack of experience of the investigators in cancer related work, a lack of the proposed clinical correlations, a lack of biostatistical consultation in the design of the studies, and the serious flaws involved in the proposed pilot studies of the clinical utility of the approach. If considered solely as an exercise in technology enhancement this proposal could have ranked in the outstanding range. This program project is rated at a very good to excellent level of merit.

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## OVERALL DESCRIPTION (Applicant's description)

The long range objective of this proposal is to develop sensitive and specific approaches to the detection and simultaneous identification of cancer-related, genetic alterations. Mutations and genetic aberrations have been implicated, at various steps, in the etiology and biology of tumors. Inherited mutations account for the predisposition to cancer in some families. Somatic mutations in tumor suppressor genes, oncogene amplification and viral DNA sequences have been found in cancers as well. However, the clinical use of these discoveries and research into their clinical significance has been slowed by the laborious process by which they are detected. To apply these discoveries and explore the interactions of multiple genetic alterations, we urgently need a new technology, which is capable of being automated and has the power to detect any of a vast number of mutations.

In response to the urgent need for new methods of mutation detection, we have assembled a team of investigators whose expertise will be directed toward innovative solutions to this problem. The collaborative nature of the scientific and organizational infrastructure will facilitate the attainment of the projects' specific aims and objectives.

The specific aims of the five projects in this program project are to: (i) develop a multiplex polymerase chain reaction/ligase detection reaction (PCR/LDR) system for the detection of inherited mutations in germline DNA and somatic mutations in tumors; (ii) develop a ligase detection reaction/polymerase chain reaction (LDR/PCR) system for detecting gene amplifications and deletions in tumors; (iii) develop a PCR/restriction /LDR (PCR/RE/LDR) system for detecting and identifying mutations in rare cancer cells at a sensitivity of 1 in 10<sup>6</sup> or 1 in 10<sup>7</sup> by removing normal DNA sequences and selectively amplifying cancer mutations; (iv) design and synthesize nucleotide analogues for converting specific DNA sequences into restriction endonuclease recognition sites for PCR/RE/LDR mutation detection; (v) engineer a thermostable ligase with greater fidelity to enhance LDR and LCR specificity; (vi) design and synthesis oligonucleotide or peptide nucleic acid (PNA) addressable arrays for the simultaneous detection of multiplex LDR and LCR products; and (vii) explore the ability of these technologies to further our understanding and clinical management of lung, colon, breast and cervical cancers.

#### OVERALL CRITIQUE

The goal of this program project is to develop sensitive PCR/LCR and very sensitive PCR/RE/LCR base substitution mutation tests which could be used to screen, in multiplex, large numbers of tumors for important mutations related to cancer (PCR/LCR) and to screen large numbers of cells for the presence of these mutations (PCR/RE/LCR). The technology development effort in Project 1, together with Project 2 could be outstanding, although the separation of the two projects appears artificial.

The enthusiasm for Project 1, led by Dr. Wilson, is tempered by an inadequate discussion of potential false positive results. More important, is the weakness of the proposed clinical correlations. The experimental plan for these correlations is diffuse and poorly described. The screening of 90 tumors by PCR/LCR does not permit sufficient statistical power to allow for any correlations with clinical outcome and no acceptable plan is put forward to achieve this end. Given that the diagnosis of recurrence in lung and colon cancer requires examining known sites of metastases (e.g. liver, bone ) and the inability of current treatment to significantly impact on survival after documentation of recurrence, it is not clear how PCR/RE/LCR would permit early detection of metastases in a useful way. A focus on the prognostic significance of micrometastatic disease at the time of diagnosis or on the detection of known mutations in cells not yet cytologically "malignant" would have far greater utility. It is not clear what the second clinical site in Denver adds to the overall project. The feasibility of the proposed technologies could be tested adequately on the samples already located in New York. Overall, this project is rated as very good.

Project 2, headed by Dr. Francis Barany, is considered to be the stronger of the two clinical projects and stronger enthusiasm is expressed for the application of the proposed technology to the problems inherent in working with clinically

heterogenous tissue such as that found in human breast cancer. As in Project 1, there is considerably less enthusiasm for the clinical-correlative studies. The investigators did not present a convincing case that they understood the extent of the clinical base of materials available and whether they knew how to apply their assays even in a preliminary way. The project would have benefitted from the up-front collaboration of a biostatistician. The studies proposed for cervical cancer, although interesting, are not considered to add anything to the project and could be dropped. Overall, the project is rated as excellent.

Project 3 is directed by Dr. Donald Bergstrom. The proposed design and synthesis of candidate convertides and universal bases for development of new and useful oligonucleotide diagnostics represent a largely empirical, albeit scientifically sound and potentially highly significant, effort. The structures and synthetic routes appear reasonable. Promising results have already been obtained and it appears that the proposed iterative "synthesis-testing-synthesis" scheme is feasible. In view of the challenging synthetic goals and the lack of a convincing rationale for the propenyl oligonucleotides, it is recommended that this latter element be reconsidered. The potential for this project to have a significant impact on nucleic acid based technology leads to an overall rating of outstanding.

Each of the three specific aims proposed in Project 4, led by Dr. Francis Barany, has the potential to generate important information about the Tth DNA ligase in particular, and DNA ligases in general. With respect to the applicability to the rest of the program project, the proposed determination of the structure of the ligase is considered to be the weakest part of the project. The probability that the structure will actually be solved early enough in the project to be applicable to the other projects is considered remote, although the work itself is of major importance to the field. The ability of an improved ligase to enhance the sensitivity of the LCR/LDR assays, and hence their potential diagnostic value leads to an overall rating of excellent for this project.

In order to carry out the large-scale screening of mutations, the Project Leader, Dr. George Barany (Project 5), proposes to develop spatially addressable arrays of oligonucleotides or peptide oligonucleotide analogs. Ligase reaction products will be constructed with fluorescent groups and will bear specific "zip code" tails. The tails will be selectively captured by complementary zip code probes immobilized in the array. Each zip code will map for a specific, known, genetic mutation. Serious concern is expressed over the ability of this workplan to actually deliver a working array to the other projects. The project lacks adequate planning in hybridization, signal to noise ratios, array fabrication expertise and instrumentation development. On the other hand, the concept of zip code sequences and the potentially elegant contributions to solid-phase chemistry by the project leader are considered strong assets. Also recognized, is the broad applicability of the technology to areas outside of cancer diagnostics. Overall, the project is rated as very good to good.

Core A, headed by Dr. Niel Hackett, proposes to: (1) create and maintain a relational database from existing clinical databases in New York and Denver; (2) perform statistical correlations of clinical outcomes with laboratory studies; (3) develop software for the selection of appropriate primers and zip codes; and (4) continue support of both instrumentation and connectivity. All four elements of this facility are considered essential; however, the experience of the Core Leader is considered

sufficient only for elements 3 and 4. There is no demonstrable expertise in the acquisition or analysis of clinical data and this is a major weakness of the overall application. Thus a merit rating of this Core is at acceptable level of merit.

Core B (Dr. Francis Barany) is recognized as an essential part of this program project. The reviewers consider that the primary function of this core should be to provide oligonucleotide reagents to the rest of the program, and to a lesser extent, provide a testing service for polymerase fidelity and efficiency of nucleotide conversions. This Core would benefit from additional expertise in the areas of fluorescence instrumentation and the implementation of robotics. Overall, this core is rated as excellent.

Core C, directed by Dr. Francis Barany addresses the need to coordinate the interaction of the applicant organization with six separate consortium institutions. The projects are proposed to operate largely independently, with this core focusing on communication and reporting requirements. Dr. Barany has demonstrated an ability to develop substantive scientific collaborations, but evidence of administrative systems to promote and monitor these interactions is lacking. Dr. Buck, the administrative co-investigator, has been recruited on a part-time basis from the Strang Clinic to address these systems but his contribution to-date is not evident. An impressive panel of external advisors has been recruited, but the means to internalize their advice and maximize the efficacy of an annual review have not been addressed. A vast number of letters of support are provided that propose specific studies unrelated to this grant while providing strong support for the overall concept. The overall rating for this core is good to acceptable.

Program as an Integrated Effort: Although this program is well integrated and has synergy as far as its goals are concerned, the diverse locations of the laboratories along with a lack of a plan to hold the frequent meetings between the project leaders, on the part of Dr. Francis Barany, hinder the interactions between the investigators. However, formulation of a plan by the Principal Investigator to hold bimonthly meetings could facilitate such efficient interactions.

#### PRINCIPAL INVESTIGATOR

Dr. Barany received his Ph.D. degree in Microbiology from The Rockefeller University in 1981 where he also spent a year for a postdoctoral training. He then moved to Dr. Hamilton Smith's laboratory at The Johns Hopkins University for a three year postdoctoral training in Molecular Biology. He returned to New York in 1985 to assume a faculty position at the Cornell University Medical School where he is currently an Associate Professor of Microbiology. He is also an Adjunct Associate Professor at The Rockefeller University. His scientific productivity is reflected by his numerous publications in highly competitive refereed journals. However, Dr. Barany clearly expresses a lack of experience in dealing with the problems posed by human cancer and describes a series of existing and potential collaborations to overcome this issue. He does not demonstrate previous experience in managing a project of this magnitude but his ability to pull together and provide leadership to this program has led the reviewers to believe that he is well qualified to serve as the Principal Investigator.



## SUPPORT TO BE NEGOTIATED FOR REPLACEMENT

InvestigatorGrant Number

F. Barany

USAMRDC Pending ("Multiplex Detection of Point Mutations, Amplifications, and Deletions in Breast Cancer")

M. Lubin

USAMRDC Pending ("Multiplex Detection of Point Mutations, Amplifications, and Deletions in Breast Cancer")

INDIVIDUAL PROJECTS AND CORES

Project 1: Genetic Markers of Lung and Colon Cancer  
(Vincent Wilson, Ph.D.)

Description: (Applicant's description) In the last decade mutations in many oncogenes and tumor suppressor genes have been described in cancers. This knowledge, however, has not significantly changed the care of cancer patients. Do cancer mutations predict the behavior of tumors? To correlate mutations with clinical outcomes we need robust methods to identify many possible mutations. Can the early spread of cancer be determined by finding the mutations of cancer cell in the bone marrow? To detect micrometastases or early cancers we must be able to detect a few cancer cells out of many normal cells.

To achieve these capabilities we have devised two technologies: polymerase chain reaction/ligase chain reaction (PCR/LCR) to survey tumors for a wide number of mutations simultaneously; and polymerase chain reaction/restriction endonuclease digestion/ligase chain reaction (PCR/RE/LCR) for detecting a few cancer cells out of many normal cells. When they are fully developed PCR/LCR should be able to detect tens to hundreds of mutations at a sensitivity of one in 10<sup>2</sup> or 10<sup>3</sup>. PCR/RE/LCR has already detected one mutation-bearing cell out of 10<sup>7</sup> normal cells.

To demonstrate the feasibility of these methods our specific aims are to: (i) Develop a PCR/LCR multi-gene, multi-mutation detection system to simultaneously identify mutations in three condons of the k-ras oncogene and nine condons of the p53 tumor suppressor gene. Approximately half of colon cancers have these k-ras mutations. About 15 percent of lung tumors and about 21 percent of colon cancers have one of these nine p53 mutations. Using PCR/LCR to identify these mutations we will investigate 40 colon and 50 lung tumors; (ii) Refine PCR/RE/LCR to detect the above p53 mutations at sensitivities of one in 10<sup>7</sup>. We will first use PCR/RE/LCR to determine the natural background mutation rate in non-cancerous tissues. Then, for patients whose tumors had detectable p53 mutations, we will use PCR/RE/LCR to investigate lymph nodes, blood and bone marrow specimens for micrometastases.

Critique: The project leader proposes to amplify genomic fragments from cancer-related genes using PCR and to rely on the allele specificity of the ligase chain reaction (hence PCR/LCR) to detect specific point mutations. Additional selection

is proposed to be added during the PCR amplification by way of restriction endonuclease cleavage of the wild type sequences (PCR/RE/LCR).

The project leader makes a compelling case that LCR (or LDR in Project 2) is a good method for detecting mutations in clonal tumor populations or in tissue samples in which the tumor cells represent 0.1-1 percent of the cells. Ease of multiplexing was put forward as the major advantage of LCR over allele-specific PCR. Nevertheless, unlike LDR in project 2, no preliminary data is presented for PCR/LCR with  $N > 2$ . For the work scheduled for years 1-3, the project leader has selected mutations in 3 codons of k-ras (20 mutations) which will detect mutations in about 50 percent of colon and an undisclosed number of lung cancers, and mutations in 9 codons of p53 found in 15 percent of lung and 21 percent of colon cancers. PCR/LCR would be applied to 40 colon and 50 lung tumors from a more than adequate supply of available specimens. It is not clear how these specimens would be selected. No power calculations are given to assess the effort needed to extract clinically relevant data (e.g. prognosis) from this type of survey. It is also disappointing that no calculation is given as to the effort necessary to develop a multiplex PCR/LCR system capable of detecting mutations in a large fraction (e.g. 95 percent) of either lung or colon cancers, a prerequisite to screening or other applications of PCR/RE/LCR. Also, the discussion of the use of readily available fixed specimens is inadequate.

The project leader has also made a compelling case that PCR/RE/LCR could be developed in years 2-5 into a highly sensitive method for detecting mutations in tissue samples where tumor cells represented approximately 1/10<sup>6</sup> cells. The competing technology of PCR/RE/allele-specific PCR is not compared, although the previous advantage of potentially easy multiplexing in PCR/LCR would be lost in PCR/RE/LCR. No explanation was proffered as to why dilution of mutant plasmid into wild type plasmid led to decreased signal intensity (p. 218 of the application) whereas dilution of 1-10 cells containing mutant DNA into wild type cells led to constant signal intensity (p. 220). Furthermore, the project leader stated that experiments with 1 cell are routinely positive. Given the required sensitivity and discrimination of these experiments, data showing detection as a function of PCR cycle number for the various amplifications would have been reassuring. Although he is an expert at applying PCR technology, the discussion of contamination is inadequate for the task at hand. PCR/RE/LCR is equivalent to sperm typing, where heroic efforts have been needed to eliminate false positives from amplification product carry over.

A discussion of potential false positives due to DNA polymerase errors is included, but the assumed DNA polymerase error rate is at least an order of magnitude lower than the cited literature. Ligase may be contributing of the order of 100-fold to the discrimination. Nevertheless, the results (Fig. 4 & 6) for PCR/RE/LCR at MspI sites, which contain only dG and dC and which are copied by Taq polymerase with greatest fidelity, might suggest that the same degree of PCR/RE discrimination could not be achieved for the majority of mutations.

The proposal to examine various clinical samples for micrometastases, except as applied to establishing initial staging, appears to be misdirected to tissues unrelated to metastatic spread.

Personnel:

Name: Vincent Wilson

Degree/Discipline/Date: Ph.D., pharmacology & toxicology, 1980

Role/Percent Effort: Project Leader, 20 percent

Qualifications/Experience: Dr. Wilson was a Senior Staff Fellow in the Laboratory of Human Carcinogenesis, NCI (1982-1988) where he published extensively with Dr. Curtis Harris on DNA adducts. His recent and independent publication record has not been particularly impressive. The work on which this study is based derives from his SPORE pilot project and his collaboration with the applicant. They have developed a highly sensitive method for detecting point mutations in one cell in millions, which if generalized could be important for cancer biology. Dr. Barany credits Dr. Wilson with the original concept of the application.

Assessment in designated role: highly qualified

Name: Leonid L. Reznikov

Degree/Discipline/Date: M.D., andrology and urooncology, 1986; Ph.D., laser medicine, 1990

Role/Percent Effort: Postdoctoral fellow, 100 percent

Qualifications/Experience: His recent publications, in Russian, have all been in urology. He has no background in molecular biology.

Assessment in designated role: qualified.

~~Budget:~~ The modest budget is approved as requested.

Assessment: Level of merit; very good

Project 2: Genetic Markers of Breast and Cervical Cancer  
(Francis Barany, Ph.D.)

Description: (Applicant's description) To improve cancer care, researchers and clinicians need robust methods of identifying genetic alterations in cancers. There are three important challenges that need to be met: (i) the detection of many possible point mutations in tumors; (ii) the quantification of gene amplifications and deletions in tumors; and (iii) the detection of rare cancer cells against a background of normal cells. Researchers need these capabilities to be able to correlate multiple genetic alterations with clinical outcomes, identify new cancer-related genetic loci, and detect early cancer recurrence and premalignant cell.

To accomplish this, we will develop: (i) a multiplex polymerase chain reaction/ligase detection reaction (PCR/LDR) system to detect many possible point mutations in cancers; (ii) ad multiplex ligase-detection reaction/polymerase chain reaction (LDR/LCR) system to quantify gene amplifications and deletions in tumors; and (iii) a polymerase chain reaction/restriction endonuclease/ligase detection reaction (PCR/RE/LDR) to identify 1 cancer cell in 106 normal cells.

Some issues in cancer will be explored. Specifically we will: (i) Expand PCR/LDR to detect 24-40 point mutations, (63% to 79% of p53 gene mutations) in breast tumors. PCR/LDR will also be used to detect high risk human papillomavirus (HPV) in cervical

lavages or biopsies. (ii) Use "zip code" primers to proportionally PCR amplify and quantify LDR products of genes deleted or amplified in tumors. This should allow us to simultaneously detect HER-2/neu and int-2 gene amplifications, as well as p53 gene deletions in breast tumors. (iii) Refine PCR/RE/LDR for detecting five p53 mutations by selectively amplifying mutated DNA while removing wild-type products by TaqI restriction endonuclease cleavage. We will then characterize p53 gene mutations and deletions, HER-2/neu amplifications, and int-2 amplifications in 100-200 frozen breast tumors and corresponding fixed specimens. We will use PCR/RE/LDR to look for micrometastases in the bone marrow and lymph nodes of patients whose tumors had one of the five specific p53 mutations. Ultimately, this sensitivity method may identify early relapses or primary tumors by detecting circulating cancer cells in the blood.

Critique: In Specific Aim 1, a multiplex PCR/LDR system will be developed to detect mutations in the p53 gene and to detect high risk HPV strains in clinical samples. The strength of the project is that rapid methods to detect single base pair mutations are much needed in the clinical arena, and the PCR/LDR and the PCR/RE/LDR methods may prove useful for this purpose. From a technical point of view, the boundaries between this project and Project 1 are artificial; this project is distinguished from Project 1 primarily by the fact that Project 1 concentrates on LCR methods while this project concentrates on LDR methods. The three specific aims entail PCR/LDR of tumor biopsies and Human Papilloma Virus (HPV), LDR/PCR for measuring gene amplification, and PCR/RE/LDR for detecting mutations at a sensitivity of 1 in  $10^6$  or  $10^7$ . In contrast to project 1, the target genes are directed towards genes involved in breast cancer (and HPV infection associated with cervical cancer), with an overlap with project 1 in regard to detecting some p53 mutations. The project leader hopes to identify new correlations with prognosis and the mutations revealed.

The project leader has chosen to initially examine five different mutation sites within the p53 gene (constituting about 28% of reported p53 mutations) because these are known sites for TaqI conversion using PCR/RE/LDR. This specific aim proposes to develop the method for large screen testing using a pilot panel of 100 to 200 breast cancer cases from frozen or fixed specimens. However, no preliminary data was shown using a sample with a known mutation at one of these sites in either frozen or fixed breast cancer samples. Furthermore, it is unclear from the letters of collaboration, the nature of the corresponding follow-up clinical information on these patients. The letter of collaboration from Dr. Kovach states that 75 known p53 mutation sites-containing samples would be made available, but it is not clear which of these contain the five p53 mutations chosen for study. The letter from Dr. Summers does not state any sample information, but appears to be referring to the wrong grant application. However, it is apparent that the well-characterized tumor bank available through Dr. Osborne is a valuable resource which will be made available to the investigators.

This project refers to project 4 because improvements in the buffer conditions or ligase may be needed in order to increase the sensitivity of PCR/LDR to 1 cancer gene mutation in  $10^2$  to  $10^3$  normal cells. If one accepts that the need for further optimization of pH, salt concentration, incubation time and temperature that give maximum fidelity for Tth ligase in LDR assay, and thus LCR (see page 356 of project 4), then the experiments proposed seem premature. It is most important to optimize the signal before performing complex experiments.

Another issue is that the investigators have chosen the p53 gene because of the frequency with which mutations have been detected in tumors, and it is a valuable test system for developing the methods of PCR/LDR and PCR/RE/LDR. But it is questionable whether the investigators will be able to correlate their findings due to their current lack of integration with the clinical database and their lack of statistical power. Even very good prognostic markers with hazard ratios of 1.3 to 1.4 and rare mutations rates (for instance of 8 percent) would require 1000 to 1700 specimens to see differences in disease free survivals (in node-negative disease) after five years. Additionally, there is no discussion of statistical analysis in the project; a statistical collaborator would strengthen this project.

It is of concern that cellular heterogeneity inherent in breast cancer has not been adequately addressed; this may be problematic in PCR/LDR assays. It would probably be best to develop Specific Aim 3 first, and increase sensitivity, before screening large numbers of samples outlined in Aim 1.

The project leader has also included studies to detect high risk HPV strains in cervical carcinomas. It is felt that the inclusion of this study, when the rest of the project is devoted to the detection of breast cancer-specific changes, makes the project diffuse. It is suggested that this study be omitted.

The arguments for gene quantification as a method to look for ploidy changes are quite convincing. However, figure 7 in the application is a multiplex experiment in which the ratios should be 1:1 but they are not; this is not discussed by the Project leader. It is stated that "the prognostic significance of gene amplifications (e.g. HER-2, c-myc, and int-2) in breast cancer has not been clearly established..)". There are numerous studies demonstrating the clinical utility of HER-2 in node-positive disease, and the lack of utility of int-2 as a prognostic marker. It is not understood why int-2 has been chosen for study due to its low level amplification rate in breast cancer (less than 15 percent) and due to the fact that the protein product of int-2 is not expressed in breast cancer. Although the development of an assay for gene amplification is worthy of study, the development of it for genes such as the int-2 gene is both scientifically uninteresting, and a poor choice for study. However, the HER-2 gene is an appropriate choice for methodology development if HER-2 amplification status has been previously determined by conventional methodologies in these clinical samples.

In Specific Aim 3, PCR/RE/LDR will be developed to detect rare mutations with increased sensitivity for the eventual application of occult micrometastasis identification; this aim is undoubtedly the strength of the project. The PCR/RE/LDR strategy could prove to have a relatively high sensitivity in clinical samples. As the investigators point out, the level of error incorporation of Taq polymerase is unlikely to average over 1 in  $10^6$  cells, and furthermore, the ligase has a 50x to 500x selectivity against mismatches involving the 3' nucleotide of the ligation substrate. Preliminary data would have dispelled these doubts.

To generalize PCR/RE/LDR, the project Leader suggests conversion of mutation sites to include a restriction site by the use of primers with "convertide" nucleotides or nucleotide analogs. The example given is for transitions from CCGG to TCGA, the easiest of such conversions. The investigators propose that other harder conversions could use nucleotide analogs that permit the introduction of other bases in replication (project 3).

The investigators in Project 3 may have already found a universal "convertide" for this purpose. The other proposed convertides would not now be deemed necessary for this project. They would still be interesting as variants at the penultimate 3' base for increased discrimination. It should be noted that there is no reference to previous work regarding "converting" sequences to restriction sites using PCR. These include Hruban et al., Am. J. Path., 1993 and Mitsudomi et al., 1991. These alternative technologies should have been discussed.

From the point of view of error rate in polymerization or ligation, the examples shown generally represent atypically easy targets for demonstrating this strategy; the restriction site is 5'-CCGG (or 5'-TCGA with the T and A defined by the primers) and it is known that Taq polymerase has its lowest error rate for G:C base pairs. The mutations detected are usually transversions, for which the ligase has the maximum possible discrimination. This means that the example shown will not reflect the typical combination of A:T-containing restriction sites and transition mutations that will be encountered in a big survey. The problem of fidelity at some sites was acknowledged at the visit.

Once conversion of a site is contemplated there can be no multiplexing. One should also consider whether allele-specific ASO (similarly spiked to give quantitation) would be equally efficacious, or even more so, after iterated PCR/RE. Such a PCR step could take place under maximum fidelity conditions (such as single stranded binding protein and low nucleotide concentrations) which seems to match the fidelity of LCR. Specific reasons why this is not a good idea should be given.

The investigator recognizes that in many of the cases for which this methodology may be useful, the issue will not only be that of whether a mutation is present, but also how prevalent this mutation might be. To achieve this the reaction will be spiked with a primer containing a different 3' base than the one to be assayed and with a different product length. This strategy assumes that the chosen spike sequence will never occur in a tumor but is nevertheless likely to work in many cases.

Regardless of these concerns, a high degree of enthusiasm is expressed for this study, independent of the ability of the project leader to correlate the information gained with clinical parameters.

Intrinsic Scientific Merit Score: 183

Personnel:

Name: Francis Barany

Degree/Discipline/Date: Ph.D., microbiology, 1981

Role/Percent Effort: Principal Investigator, 15 percent

Qualifications/Experience: described earlier.

Assessment in designated role: highly qualified.

Name: Matthew B. Lubin

Degree/Discipline/Date: M.D., medicine, 1984

Role/Percent Effort: Co-Investigator, five percent

Qualifications/Experience: Internship and Residency, and Fellowship in Internal Medicine, and Medical Genetics, respectively (1987-1990); Clinical Instructor (1990-1993); Director of Medical Genetics, and Assistant Professor (1990 and 1993).

Assessment in designated role: well qualified.

Name: Darren Day

Degree/Discipline/Date: Ph.D., biochemistry, 1989

Role/Percent Effort: Research Associate, 100 percent

Qualifications/Experience: Postdoctoral Fellow at the University of Southampton, United Kingdom, and Auckland University, New Zealand (1990-1992).

Assessment in designated role: qualified.

Budget: The requested budget is approved.

Assessment: Level of merit; excellent.

Project 3: Design and Synthesis of Nucleotide Analogues  
(Donald Bergstrom, Ph.D.)

Description: (Applicant's description) A high sensitivity mutation detection system must be able to detect changes in any gene sequence. To be able to accomplish this, wild type DNA sequence corresponding to a mutation needs to be converted to a restriction enzyme site so repeated PCR amplification followed by digestion with the restriction enzyme removes the normal sequence while selectively amplifying the mutant sequence. This amplification, known as PCR/RE/LDR aims to detect one cancer mutation in 106 normal cells.

The goal of this project is to design and synthesize nucleotide analogues which facilitate sequence conversion. "Convertides" are nucleoside analogues which pair to one or more of the natural bases in an initial primer hybridization. More importantly, convertides also function as a degenerate template allowing for insertion of different base during subsequent rounds of polymerase amplification. There are twelve possible nucleotide conversions which should be achieved.

To accomplish our goal we will work towards the following specific aims: (i) The synthesis of deoxyribonucleoside analogues to be used as convertides. Eight deoxyribonucleoside analogues, Q2, Q5, Q6, and Q9-Q13, have been previously described. We have already designed nine additional modified deoxyribonucleosides, Q1, Q3, Q4, Q7, Q8, and Q14-Q17. All 17 deoxyribonucleosides analogues will be synthesized in our laboratories. (ii) Preparation of dimethoxytrityl (DMT)-protect derivatives of all the convertides for incorporation into oligonucleotides. In the middle of an oligonucleotide, DMT-convertide phosphoramidites will be used. At the 3' position this will be accomplished by attaching the 3'-hydroxyl of the protected convertide to a long chain alkyl amine-CPG support. (iii) Testing of convertides for use in the mutation detection techniques. Starting with Q2, Core B will test convertide oligonucleotides as a means of increasing the specificity of mutation detection and as universal bases for polymorphic sites. (iv) Synthesizing and incorporating 5-propynyluridine into DNA or PNA "zip codes". In addressable arrays, this will be tested for optimizing the Tm of the zip codes/complementary zip code duplexes (Project 5 and Core B).

Critique: The first Specific Aim of this project is to synthesize 17 deoxynucleoside analogues (Q1-Q17) to be incorporated into convertide oligonucleotide probes and primers; 8 analogues have been previously reported and 9 are apparently new. The underlying theory for the selection or design of each of these particular analogue structures seems reasonable. The H-bonding bonding schemes and tautomeric forms shown on p.321 of the application are also reasonable, with the exception of Q4 and Q8 in which oxygen has been replaced by sulfur. It would have been useful in each of these two less familiar cases to include supportive thermodynamic calculations. The same is true for several C-nucleosides, the duplex-forming properties of which are not clear-cut. On the other hand, the applicant's rationale regarding the selection or design of Q1-Q17 is supported by his preliminary studies of Q2 that include thermal melting ( $T_m$ ) measurements to determine if Q2 is non-discriminatory in base-pairing to A, C, G, and T, as well as investigation of sequencing and PCR reactions. While the  $T_m$  resulted per se are can only be suggestive of non-discriminatory base-pairing of Q2, it is very important that an oligonucleotide with Q2 (but not with mismatches) was already shown by the applicant to be functional in primer extension in PCR and by T7 DNA polymerase even with Q2 located at the 3'-end of a primer, which is a critical locus for the proposed convertide mechanism.

The applicant's preliminary synthetic results for Q14 are likewise supportive. Known-target analogues Q12 and Q13, the synthesis of which are said to be in progress along with Q5 and Q9, have already been shown by others to be tolerated by Taq polymerase when at the 3' end of a primer. This gives this study further credence with regard to feasibility.

In summary, the structural targets proposed in the first Specific Aim represent novel and challenging design and synthesis efforts that are correctly viewed by the applicant as being an empirical screening process to find which candidate convertides will actually provide acceptable biochemical "read" and "write" kinetics. While this adds some uncertainty to the degree of success achievable with each of the 17 convertides presently proposed, there seems to be no other way to approach the problem at this stage, since even state-of-the-art molecular modeling can at best be used to assess relatively simple H-bonding schemes and duplex stability but none of the critical polymerase reactions.

Regarding synthesis, half of the targets are known and should therefore pose no significant difficulties. The new convertides involve largely conventional transformations with apparently reasonable literature precedent. These syntheses will involve a substantial amount of work but are otherwise unremarkable. Alternative synthetic schemes are said to be available, although not written in the application.

The second Specific Aim, which is the preparation of DMT-protected support-bound and phosphoramidite derivatives of the convertides, has been adequately addressed using relatively straightforward chemistry that should pose no significant problems for the applicant's team. Purification and analysis of these oligonucleotides is described only very briefly; however, again there is ample precedent for this type of post-synthetic work.

The third Specific Aim involves testing of convertides as a means of increasing the specificity of mutation detection and serving as functionally useful bases



for polymorphic sites, which is work carried out in program projects 1, 2, and 4. Promising convertides will be further studied by the applicant using conventional measurement of thermodynamic parameters.

The fourth Specific Aim concerns synthesis of 5-propynyl-dU by the Method of Froehler in order to obtain modified oligonucleotide with supposedly higher hybridization affinity and thus, in principle, obtain greater discrimination in the addressable arrays described in Project 5. A similar proposal was made for 5-propynyluracil PNA monomers with either Fmoc or Boc protection. This is an interesting idea in the case of dU based on Froehler's published initial work; however, the generality and magnitude of this effect in mixed-based sequences has not apparently been reported. There is apparently no experimental precedent that the 5-propynyl effect will apply to PNA-DNA heteroduplexes, nor is any supporting theoretical rationale developed by the applicant. Moreover, no already proven options such as 2'-O'alkyl oligonucleotides are even discussed. While this aspect of the work is far less critical to realization of the convertide concept, it nevertheless ought to be reconsidered.

In summary, this is an exciting project based upon the early work of others and preliminary promising results obtained by the applicant. It can contribute novel compounds for potentially promising and generally useful enhancements to hybridization-based detection and a justification schemes proposed in the program and possibly beyond. The synthesis of convertide-containing oligonucleotides by the present project is clearly critical for the success of the overall program. A specific task time-table is not developed indicating who is to make which convertides by specified projected dates for delivery to the other projects and core components; however, the synthetic work is distributed between Drs. Bergstrom and Hammer according to the heterocyclic ring size, and substantial progress is said to have been made. In view of the challenging scope of synthesis, and the importance of this project to the program, it will be important for the project leader to closely monitor and direct as needed, the work that is proposed to be conducted in Dr. Hammer's laboratory. This project can conceivably have far reaching and substantial impact on nucleic acid-based technologies beyond the scope of the program.

Intrinsic Scientific Merit Score: 139

Personnel:

Name: Donald E. Bergstrom

Degree/Discipline/Date: Ph.D., organic chemistry, 1970

Role/Percent Effort: Project Leader, ten percent

Qualifications/Experience: Professor of Medicine and Chemistry at Purdue University since 1989 and Deputy Director of the Purdue Cancer Center since 1992; he has served on the editorial board of Nucleosides and Nucleotides since 1992 and has a productive publication record in synthetic aspects of that field.

Assessment in designated role: highly qualified.

Name: Robert P. Hammer

Degree/Discipline/Date: Ph.D., organic chemistry, 1990

Role/Percent Effort: Co-investigator, ten percent during nine month academic years/66.7 percent during three month summer

Qualifications/Experience: Postdoctoral 1990-1992 at ETH with world-renown Professor Albert Eschenmoser on project-related chemistry, and Assistant Professor of Chemistry at LSU since August 1992; he has only one relevant publication in J. Org. Chem. in 1987 and several proceedings abstracts.

Assessment in designated role: qualified

Name: Guanygi Wang

Degree/Discipline/Date: Ph.D., organic chemistry, 1987

Role/Percent Effort: Postdoctoral Fellow, 100 percent

Qualifications/Experience: Postdoctoral positions at the University of Arizona, University of Maryland, and Purdue University, the latter since 1989 with Dr. Bergstrom; he is listed as a co-author on about a dozen organic chemistry journal publications since 1987.

Assessment in designated role: qualified.

Name: Peiming Zhang

Degree/Discipline/Date: Ph.D., organic chemistry, 1980

Role/Percent Effort: Research Scientist, 50 percent.

Qualifications/Experience: Postdoctoral since 1990 with Dr. Bergstrom; he is co-author of only one publication, which is with Dr. Bergstrom on the synthesis of a relevant nucleotide analogue.

Assessment in designated role: qualified.

Name: Melissa Cothorn

Degree/Discipline/Date: B.S., chemistry, 1993

Role/Percent Effort: Research Assistant, 100 percent at no cost

Qualifications/Experience: No publications

Assessment in designated role: no proven experience.

Budget: The requested budget is modest and is approved.

Assessment: Level of merit; outstanding.

Project 4: Engineering an Improved Thermostable Ligase  
(Francis Barany, Ph.D.)

Description: (Applicant's description) One of the fundamental problems in detecting cancers in tissue samples is the need to distinguish a few cells containing the cancer mutation from the vast majority of normal cells. We have developed a novel polymerase chain reaction/ligase detection reaction method (PCR/LDR) for high throughput, low sensitivity mutation detection ( $1$  in  $10^2$  to  $10^3$ ), and a PCR/restriction endonuclease/LCDR (PCR/RE/LDR) method for high sensitivity mutation detection ( $1$  in  $10^6$  to  $1$  in  $10^7$ , see Project 1 and 2). The enzyme which provides the specificity for these methods is Tth ligase, the gene for which was originally cloned in our laboratory. The limit of detection of these two methods would be significantly improved by increasing the specificity of Tth ligase.

We are developing a comprehensive approach to understanding the mechanism of Tth ligase action, and improving its fidelity for discriminating perfectly matched from mismatched substrates. The three parts to this program are: (i) Developing a rapid assay to test different reaction conditions, mutant Tth ligases, and demonstrates higher sensitivity then the discriminating base is on the 3' end of the test primer. Introducing a nucleotide analogue (see Project 3) or mismatched base adjacent to or near the discriminating base may increase the specificity of this reaction. Such modified oligonucleotide primers will be tested in our fidelity assay using wild type and mutant Tth ligase. (ii) Determining the 3-dimensional structure of Tth ligase-DNA complex. This structure will help to reveal the mechanism of DNA ligation, and provide an understanding of the specificity of the enzyme for mismatches at the nicked site. (iii) Using site-specific mutagenesis to construct mutant Tth ligases. Design of these mutants will be based on protein sequence homology and protein-sugar-phosphate backbone contacts as determined from the X-ray structure. We have already isolated and partially characterized over 30 site-specific Tth ligase mutants, and these will be tested in our fidelity assay.

Critique: The overall goal of this project is to better understand the functional domains of Tth ligase and its mechanism of action which will hopefully result in an enzyme with improved specificity. Although DNA ligases from both prokaryotes and eukaryotes have been studied for many years, there has not been a comprehensive attempt using modern methods of site directed mutagenesis to study their reactions.

The primary reason for undertaking this program is the LCR and a variation LDR which hold great promise as diagnostic methods for detection of specific mutations. The project leader is one of the inventors of LCR. Thermostable ligases give superior performance in LCR and if the specificity of the ligase could be increased the sensitivity of detection of mutations could be increased. In as much as LCR or variations of it are central to the cancer detection studies proposed in this program, this section is central to the entire program since increasing the sensitivity of LCR and LDR is seminal, particularly if multiplexing is to be done.

The construct of new mutants are based on the previous mutagenesis studies by the project leader. These studies have led to identification of the adenylation site of Tth ligase in the vicinity of residue 118. These studies confirm a conserved motif, KVDG, in Tth ligase, suggested by the other workers, to be important in adenylation. Other residues are also identified in these studies which are necessary for ligase activity but not for adenylation or deadenylation. The ability of the cloned thermostable Tth ligase to complement an E. coli host containing a temperature sensitive mutant ligase permits a very nice in vivo assay for functionality. Also, the thermostability of the Tth ligase makes possible a relatively simple partial purification procedure for the ligase which permits in vitro characterization of the reactions of the mutant enzymes. Finally, a sensitive fluorescent assay for ligase fidelity has been developed using an Applied Biosystems (ABI) DNA sequencer employing the ABI Genescan software which permits the detection of products in the range of 100 attomoles. Preliminary studies have already led to the interesting observation that Tth ligase shows greater specificity for perfectly matched substrate over mismatched substrate when the mismatch is on the 3' side of the nick.

Rationale for the design of mutants, particularly in the absence of a structure, is a key issue. At this time, the project leader has only sequence comparisons and consideration of conserved amino acids to go on. It is noted that no consideration seems to have been given if large numbers of mutants were to be

screened. This might only be possible by genetic means. Considering the biochemical assays available, which make use of the temperature stability of Tth ligase, only up to several thousand of mutants could be screened in a reasonable time but not millions. However, the project leader's success on two recently isolated mutants that show improved specificity for TG mismatches suggest that it is not too difficult to isolate relevant mutants. In any event, this is not considered a serious criticism since the Site Visit Team has a great deal of confidence in the project leader's ability to recognize and deal with this problem if it occurs.

Overall, the proposed site-directed mutagenesis studies are excellent. Clean and sensitive assay methods have been developed that should, at the very least, lead to much important information about the functional domains of Tth ligase and appear to have a good probability of yielding mutants with increased specificity for at least some mismatches.

The second Specific Aim entails the testing of modified oligonucleotides for improved specificity during ligation. The approach is to test the effect of these modifications on the specificity of ligation when they occur adjacent to and one base over from the discriminating base. If modifications can be found that increase this specificity, then potentially the sensitivity of LCR/LDR, and hence their diagnostic value would increase. To be tested are base transposing agents called "convertides" which are modified so that they have ambiguous hydrogen bonding properties which permits them to base pair with more than one base and in some cases with each of the natural DNA bases (A, T, G and C). The project leader appears to be interested in 1-(2'-deoxy- -D-ribofuranosyl)-3-nitropyrrole, abbreviated Q2, which can pair with all of the natural bases. It is noted that many other "convertides" are proposed to be made in Project 3 although it is not clearly stated whether any of these other than Q2 will be tested in this project. These studies rely on supply of the analogues by Drs. Bergstrom and Hammer, from Project 3. At the Site Visit, it was reported that Dr. Bergstrom had just delivered to the project leader a new modified nucleotide, Q18, not discussed in the application, which will be tested.

The approach taken by the project leader in studying these particular types of modified nucleotides is derived from the observations of others that allelic specific PCR amplification is enhanced by destabilizing bases near the 3' end of a primer. Most notable here is perhaps the work of Dr. Thilly at MIT in developing "MAMA" technique (MAMA - mismatch amplification mutation assay). The project leader states that "This concept of destabilizing the enzyme-nucleic acid complex to significantly increase the specificity of a reaction is the basis behind our site-specific mutagenesis studies [discussed in several sections of the application]". In the reviewers's opinion, the project leader makes a large leap in coming to this conclusion. Dr. Thilly's work used one or two natural bases at the 3' end of a PCR primer which were mismatches of bases on the template. This is quite different from the "convertides" to be used here which have ambiguous hydrogen bonding properties that allow them to pair to more than one of the natural bases. There is no way to predict the effect of these "convertides."

The general approach of testing the effect of modified nucleotides on ligation specificity is strongly supported. However, the proposed approach might be too narrowly focused on the Q2 nucleotide and the specific observation mentioned above that destabilizing bases near the 3' end of a primer enhances allelic specific

PCR amplification: It is difficult to predict the effects of almost any modified nucleotide. This section of the project might be strengthened by a broader approach including types of nucleotide modifications other than the "convertides" proposed. At the very least, "convertides" other than Q2 should be incorporated into these studies as soon as possible. Q2 was designed to be a universal nucleotide and a part of this design was to make it lack a hydrogen donor site and maximize stacking interactions (see Project 3). All of the other "convertides" proposed have a different design principle in that they all have a hydrogen donor site. It is not possible to predict how important this difference might be. In the reviewer's opinion, it should be addressed as soon as possible.

Determination of the three dimensional structure of Tth ligase is clearly an important problem. The actual work will be done by Dr. Aneel Aggarwal who has the experience to perform this study. No one can predict the difficulty in determining a protein structure. Even if the structure is not solved, this project can yield important information. However, the value of the information would increase greatly if the structure were solved, especially early on.

Intrinsic Scientific Merit Score: 145

Administrative Note: Attention is drawn to the potential scientific overlap between the proposed studies by Dr. Francis Barany and those funded at Applied Biosystems Inc., on "Ligation Amplification Technology" (2/1/92-1/31/97).

Personnel:

Name: Francis Barany

Degree/Discipline/Date: Ph.D., microbiology, 1981

Role/Percent Effort: Project Leader, ten percent

Qualifications/Experience: Dr. Barany was a postdoctoral fellow with Dr. Hamilton Smith at The Johns Hopkins from 1982-1985. He joined the faculty of Cornell University Medical College in 1985 as an Assistant Professor and currently holds the rank of Associate Professor. He is the author of numerous publications relevant to this Project. His research accomplishments include cloning of Tth ligase; he is one of the inventors of the Ligase Chain Reaction.

Assessment in designated role: highly qualified

Name: Aneel K. Aggarwal

Degree/Discipline/Date: Ph.D., biophysics, 1984

Role/Percent Effort: Co-Investigator, ten percent

Qualifications/Experience: Dr. Aggarwal was a postdoctoral fellow with Stephen Harrison at Harvard university. In 1989, he became Assistant Professor of Biochemistry and Molecular Biophysics at Columbia University. Dr. Aggarwal's postdoctoral research on the structure of phage 434 repressor-DNA complex and his more recent work since coming to Columbia University on the structure of BamHI, appear to make him well suited to lead the structural studies outlined in this project.

Assessment in designated role: well qualified

Name: Jianying Luo

Degree/Discipline/Date: Ph.D., biochemistry, 1992

Role/Percent Effort: Research Associate, 50 percent

Qualifications/Experience: has constructed, isolated, sequenced, and characterized proteins from the mutants of Tth ligase gene. Furthermore, she has performed all the ligase fidelity assays described in the preliminary results.

Assessment in designated role: qualified

Budget: appropriate as requested.

Assessment: Level of merit; excellent.

Project 5: Design and Synthesis of DNA and PNA Arrays  
(George Barany, Ph.D.)

Description: (Applicant's description) The goal of this program project is to develop methods for identifying multiple gene mutations in cancers. For maximum utility, these methods must be able to recognize and discriminate between dozens or hundreds of mutations.

To accomplish this, we propose to capture specific ligase detection reaction (LDR) products on a spatially addressable array, such that the position of a signal identifies a mutation. Each LDR product will have a "zip code" tail, which will be selectively captured by a "complementary zip code" on a solid support. The complementary components can be DNA oligonucleotides or peptide nucleotide analogues (PNA). PNA/DNA hybrids have significantly higher  $T_m$  values than DNA/DNA hybrids. Incorporation of the nucleotide analogue, 5-propynyluridine, into DNA zip code and PNA address sequences will further increase and optimize  $T_m$  values (Project 3). Unreacted LDR primer may therefore be washed away at high temperatures allowing for a higher sensitivity in detecting LDR products. A reusable, universal addressable array could be used for detecting a wide range of cancer mutations, genetic diseases and infectious agents.

Implementation of these concepts, with the ultimate goal of achieving reliable and efficient materials and procedures that can be incorporated into easy-to-use, automated, low-cost diagnostic devices, will follow these aims: (i) Development and evaluation of solid support materials compatible with chemical synthesis of DNA oligonucleotides and PNA oligomers, and compatible with subsequent hybridization reactions. Surfaces, beads, or membranes will be functionalized, and extended as needed with hydrophilic spacers such as heterobifunctional polyethylene glycol (PEG) and/or carbohydrates. Chemistry for linking oligomers to the solid support, and/or solid-phase assembly of oligomers, will be developed. (ii) Establishment of methodology for synthesis of spatially addressable arrays of DNA oligonucleotides and PNA oligomers. Appropriate masking technology will expose defined regions of the solid support for attachment of pre-formed oligomers, or for chain elongation to assemble the needed oligomers. In the latter mode, segment condensation will be used when possible in order to provide efficient convergent synthesis, and because chemical "failures" will become "invisible" during the subsequent hybridization. (iii) Demonstration of scope and limitations of zip code concepts. As aims (i) and (ii) come to fruition, testing will be carried out (Core B). Design of primer and zip code structures will be facilitated by the informatics collaboration (Core A).

Critique: In order to carry out large-scale screening of mutations, this project proposes to develop spatially addressable arrays of oligonucleotides or peptide nucleotide analogs. Ligase reaction products from the technology devised in Projects 1 and 2, will be constructed with fluorescent groups, and will bear specific "zip-code" tails. The tails will be selectively captured by complementary zip-code probes immobilized on the array. Each zip-code will map for a specific known genetic mutation.

~~The concept of zip-codes, analogous to the multiplex detection scheme of Church and Gilbert, has been very well developed in this study. The codes are conceived to be unique 24-mers, designed to have very little similarity overlap. A novel block synthetic scheme, suited to this design is proposed for PNAs. 36 of the 256 different tetramers have been chosen on the basis of minimal similarity, and will be synthesized as building blocks for 24-mer arrays. Approximately  $2 \times 10^9$  24-mers could be constructed from this building block set, out of a possible approximately  $3 \times 10^{14}$  24-mers. Initially, five tetramers have been chosen as synthetic targets for feasibility testing of the array technology.~~

Before actual arrays are to be constructed, a significant amount of DNA/PNA array development work is proposed. First, solid supports compatible with DNA or PNA synthesis will be screened. A variety of potentially compatible materials will be investigated, including glass, plastic, cellulose, PEG-PS beads and a variety of membranes. The Project Leader has a good command of solid-phase synthesis techniques, brings strength to this part of the project, and has put together an impressive list of surface functional and linker groups. In addition to the compatibility with synthesis, the hybridization compatibility of these supports will also be screened.

The investigators propose to adapt newly developed PNA chemistry to the 24-mer array format. Although PNA/DNA complexes are known to be extremely heat stable, it is not clear that this will actually help the detection discrimination issues presented in this proposed study. 24-mer DNA complexes are already quite stable, and the array design itself preselects optimal 24-mers for minimal cross-hybridization. A significant amount of chemistry must accompany the PNA array development, and although synthetically efficient and scientifically interesting, the central need for PNA arrays for achieving the goals of the program project remains unclear.

The proposed study schematically illustrates a microchannel device to construct the DNA or PNA arrays. Embedded in the actual fabrication of the arrays, is an enormous amount of engineering and development work; however, no workplan or budget is provided. During the site visit, the investigators deferred this development activity to collaborators in laboratories at Sirrus or Millipore. Unfortunately, the reviewers have no way to judge the commitment or capability of the investigators at Sirrus or Millipore to meet the priorities of this program project.

In summary, the strengths of this project lie in the novel investigations related to DNA zip-codes. The concept is well developed, designed and a synthetic procedure for efficient construction of PNA arrays is proposed. The solid-phase chemistry in this section is outstanding. However, the lack of expertise and experimental planning in hybridization, fluorescence detection, and perhaps most importantly, the inability to construct the arrays seriously weakens this section.

Intrinsic Scientific Merit Score: 244

Personnel:

Name: George Barany

Degree/Discipline/Date: Ph.D., biochemistry, mathematics, organic chemistry, 1977

Role/Percent Effort: Project Leader, ten percent

Qualifications/Experience: Dr. Barany is currently Professor of Chemistry at the University of Minnesota. He is an expert on new methods of peptide synthesis, orthogonal protection, organosulfur chemistry and various polymer functionalization chemistries. He has an outstanding publication record, and is considered to be highly qualified to carry out the synthetic and solid-phase chemistry on this project.

Assessment in designated role: highly qualified.

Name: Josef Vagner

Degree/Discipline/Date: Ph.D., biochemistry, 1990

Role/Percent Effort: Postdoctoral Associate, 100 percent

Qualifications/Experience: research worker in Prague (1986-1992) with experience in solid-phase and solution peptide synthesis, immunology, and protein chemistry. Presently, a postdoctoral fellow at the University of Minnesota.

Assessment in designated role: qualified.

Budget: The modest budget, as requested, is recommended for approval.Assessment: Level of merit; very good to good.

Core A: Informatic Support for Cancer Detection Methods  
(Neil R. Hackett, Ph.D.)

Description: (Applicant's description) The goal of this program project is to develop techniques that detect multiple cancer mutations, ultimately for the purpose of researching the relationship between genetic alterations and tumor behavior, and applying these techniques in clinical situations. Managing a database of cancer-associated mutations, developing multiplex assays for them and correlating multiple cancer mutations with disease outcomes will require a sophisticated level of data management.

Core A will provide informatics support for cancer detection according to the following aims: (i) Create and maintain a database of mutations associated with cancer, patient history and experimental results. A relational Client/Server database will be created on a central facility consisting of a SPARC station 10 running the Sybase database management system. Both published reports and results from Projects 1 and 2 will be collected. (ii) Analyze database for correlations of point mutations with clinical outcome. The significance of the cancer detection experiments in Projects 1 and 2 will be assessed by performing multivariate analysis on given mutations to determine whether they predict clinical outcome. (iii) Write programs for the choice of primers for PCR/LDR, LDR/PCR and PCR/RE/LDR protocols. The programs will be written in C language for use on IBM/PC or Macintosh computers with a simple text-base interface. These programs will aid primer design and calculation of modified primer Tm values for Projects 1, 2 and 3. (iv). Assist



in the design and analysis of oligonucleotide arrays for mutation detection. For project 5, potential schemes for array design will be explored to ensure arrays of the maximum difference in sequence between every pair of zip codes while maintaining a constant melting temperature. (v). Assist in the programming of the instruments in the diagnostics and evaluation core and interface these with the central database (for Core B). The informatics core will maintain a Client/Server database on a SPARC station, and provide programming support which is accessible to all participants in the program project. This research may lead to correlations between molecular markers and prognosis for lung, colon, breast, and cervical cancers.

Critique: This critique is based on the assessment of the investigators rather than their written or verbal description of the proposed work, since the core is vague in a number of areas.

The investigators have experience in biological sequence analysis, and together with Dr. Barany's knowledge of primer chemistry, should be well equipped to develop primer selection programs (task 3). The reviewers at the site visit asked Dr. Hackett specific questions about his strategy for this problem and received a response indicating Dr. Hackett's excellent command of this problem.

The staff has experience with instrumentation and should be able to program and interface the various instruments required by the project (task 5). However, The staff in this Core have a very limited or lack of experience in data base which is of concern to the reviewers. The application does not provides details on any aspect of the database design, not even examples of specific data that will be stored in the database, e.g., specific items of clinical history that will be stored. At the site visit, Dr. Hackett presented an example purporting to illustrate his approach to clinical data. The example was unconvincing from a clinical standpoint in that it contained no relevant clinical data. The computing aspect also revealed a serious technical flaw. (The table on the upper right hand corner contained data values that were used as column headings in the table in the lower left, violating a standard tenet of relational database design).

There is no technical discussion of the decision to use SYBASE. The proposal mentions the importance of client/server architecture which is valuable in many situations, but it is not clear that it is important in this study. SYBASE is a complicated product, and the staff appears to have no experience with it. Perhaps a simpler MaC-based product, such as Fourth Dimension, would be a better choice.

It is unclear as to why this project needs to maintain a database regarding cancer causing mutations, except for the mutations being analyzed in Projects 1 and 2. The project leaders of Projects 1 and 2 are well aware of the literature and on-going work related to the genes being studied in these projects, and are unlikely to require a database of all cancer causing mutations. The investigators may want to do a literature search to see if they have missed any new reports of relevant mutations, but this is best done by a search of MedLine and public databases using standard software.

The complex issues involved in clinical data management were not discussed at the site visit. Dr. Osborne volunteered that he and his co-workers at Strang have extensive experience in this area and would be delighted to help out. Although

this is viewed positively, this involvement should have been explicitly described in the application.

No technical discussion on how the Strang clinical database will be translated to the proposed database. It is not clear if the proposed database will use the same schema as Strang. If not, some translation effort is needed to bridge the differences.

Additionally, how data would be moved from Colorado to the proposed database is also unclear. Is the Colorado data is available on computer already, then translation issues similar to those mentioned above must be addressed. If not, substantial effort will probably be required to extract and computerize this information based on patient records.

Experience in Biostatistics area is yet another concern. The investigators provide no details on biostatistical aspect of the clinical design, nor is there any discussion of statistical methods that will be used to analyze the data. At the site visit, Dr. George Wong, a bio-statistician at Strang, expressed his enthusiasm for the proposed work, but confirmed that he has not been involved in the project to date. The involvement of a biostatistician is essential for the design of the proposed clinical studies and the analysis of the results.

Project 1 includes some discussion of data management and statistical analysis for data collected in Colorado. There seems to be no coordination between the Colorado effort and the Informatics Core. These two efforts propose to use different types of computers (Mac versus Unix), different database management systems (Paradox versus SYBASE), and different statistical packages (SYSAT and SAS versus SPSS).

Personnel:

Name: Neil R. Hackett

Degree/Discipline/Date: Ph.D., biochemistry, 1982

Role/Percent Effort: Core Leader, 20 percent

Qualifications/Experience: postdoctoral training in the Department of Chemistry at MIT; Assistant Professor in the Department of Molecular Biology at Vanderbilt University for three years, and since 1989 has been Assistant Professor in the Department of Microbiology at Cornell University. Since 1990, also served as the Manager of the Molecular Biology Computing Facility at Cornell University. He has several publications in Molecular Biology, but lists no publications related to informatics.

Assessment in designated role: qualified.

Name: Aaron Giles

Degree/Discipline/Date: B.S., physics, 1992

Role/Percent Effort: Programmer, 100 percent

Qualifications/Experience: worked as a programmer at the High Energy Physics Department, University of Chicago through June of 1993 at which time, he joined the Office of Academic Computing at the Cornell University. His CV suggests experience in software development at the hardware/software boundary, such as firmware for attached laboratory devices, print drivers, and image viewing software.

Assessment in designated role: qualified.

Budget: The operating system (\$5,000) is included in the "Equipment" category and is thus deleted from the "Supplies" category. The budget of \$2,500 in the "Other Expenses" category is not justified and therefore deleted. Due to the excessive requested budget in the years 02-05, the costs for "Equipment" (-\$2,000), and for maintenance in the "Supplies" categories (-\$1,000) are recommended to be reduced. Additionally, the cost for "Other Expenses" (-\$2,500), is recommended to be deleted as it is not justified.

Assessment: Level of merit; acceptable.

Core B: Instrumentation and Mutation Detection  
(Francis Barany, Ph.D)

Description: (Applicant's description) Correlations of multiple cancer mutations with disease outcome will require the ability to perform high throughput mutation detection. The goal of this core is to provide the instrumentation and mutation detection support required to achieve large scale identification and analysis of mutations. Core B will work closely with Core A, the informatics support for cancer detection methods.

This Core will have the following responsibilities: (i) Providing instrumentation for oligonucleotide synthesis and analysis of cancer causing mutations. The PCR/LDR, LDR/PCR, and PCR/RE/LDR experiments described in Projects 1 and 2 require synthesizing large numbers of oligonucleotides. The products from these cancer detection amplifications will be separated and quantified on an ABI 373A DNA sequencer. By the third year we plan to automate some of the PCR/RE/LDR steps using a robotics workstation. (ii) Testing the efficiency and polymerase fidelity of nucleotide conversions using convertide oligonucleotides. The PCR/RE/LDR cancer detection scheme is dependent on the fidelity of thermostable polymerase extension off primers containing a 3' nucleotide analogue (Project 3). Using an assay we developed, the Core will test both the efficiency and fidelity of different polymerases for each base conversion. (iii) Testing oligonucleotide or PNA addressable arrays for quantitative cancer mutation detection. Large scale detection of a multitude of mutations will require addressable arrays. Mutations will be distinguished by the position of a fluorescent signal on the array. The Core will test arrays synthesized in Project 5 for fluorescent detection of LDR and LCR oligonucleotide products, using a Molecular Dynamics FluorImager 575.

Critique: Core B is considered as an essential part of the program project. The specific responsibilities include providing oligonucleotides to the rest of the program, robotics support for the PCR/RE/LDR assays, testing the efficiency and polymerase fidelity of nucleotide conversions, and testing of the oligonucleotide or PNA arrays for quantitative cancer mutation detection.

With respect to oligonucleotide synthesis, an upgraded Model 394 automated 4-column synthesizer donated by ABI is available for producing the required oligonucleotides. This is now conventional technology which should not pose any difficulties. The investigators in this group have experience with gel-purified oligonucleotides, which represents valuable know-how as purity of probes and primers will be a critical issue.

Currently, products from the experiments described in projects 1 and 2 are separated and quantified on a Model 373A DNA sequencer which has also been donated by ABI and is more or less turn-key technology. The concept of using, for example, hexaethylene oxide "tails" to differentiate multiplexed PCR/LDR and related amplification products has already been proven in referenced publications 2 and 3. There is ample precedent for the use of capillary electrophoresis to achieve the proposed separations. The proposed "zip code" concept for the PNA arrays should be an effective detection scheme if implemented into arrays. The Beckman Biomek or ABI Catalyst 800 robotics equipment for pipeting, etc. seems reliable and justifiable.

In this Core, the efficiency and polymerase fidelity of nucleotide conversions using convertide oligonucleotides provided by Project 3 will be tested. The applicant has devised a series of assays to determine how well a nucleotide analogue can "read" a natural base, which were considered sufficient by the review committee.

Finally, oligonucleotide or PNA addressable arrays for quantitative cancer detection will be tested. Test arrays are to be provided by Project 5 and hybridization will be detected using a Molecular Dynamics FluorImager 575 to be purchased in year-1 for \$80,000. This system will likely require a substantial amount of unbudgeted software development. The hybridization assays will also require a substantial amount of optimization, especially if quantitation is desired. Overall, the core group could benefit from expertise in software and instrumentation development.

Personnel:

Name: Francis Barany

Qualifications described earlier under Principal Investigator and Project 4.

Assessment in designated role: highly qualified.

Name: Matthew Lubin

Qualifications described earlier in Project 2.

Assessment in designated role: well qualified.

Name: Jianying Luo

Qualifications described earlier in Project 4.

Assessment in designated role: qualified.

Budget: The modest budget is approved as requested, except that the level of effort for the research technician is recommended to be changed from six months at 100 percent effort to one year at 50 percent effort.

Assessment: Level of merit; excellent.

Core C:           Administrative Core  
                  (Francis Barany, Ph.D.)

Description: (Applicant's description) The structure of the administrative core has been designed to meet the planning and managerial needs of the program project. It is also aimed at combining the expertise of all institutional administrators and scientific program directors in order to enhance the proficiency of the research level in order to promote cooperative efforts at all collaborating institutions.

The administrative responsibility of the Administrative core will include: 1) monitoring the scientific and administrative diligence of each of the component projects and re-allocating resources when and if necessary, 3) scheduling meetings of group investigators to be held approximately three times a year, 4) to keep the NIH Scientific Program Director/Coordinator apprised of group progress, changes in scientific aims, personnel, etc. 5) preparing progress reports for the NIH 6) assuring that core resources are providing adequate support to all projects, 7) coordinating group activities with all external companies in keeping with the formal collaborative research arrangements, and 8) monitoring inventions and invention disclosures supported by the program. In this fashion, the Administrative core will enhance maximum coordination between investigators participating in the program and facilitate the research objectives of the program project.

Critique: Dr. Barany's past research accomplishments demonstrate his ability to administer effectively individual RO1 projects. Although his record of administrative experience other than that required for individual projects is only recent and limited, his success to date in assembling the program project key investigators is evident. His collaboration since 1991 with Dr. Wilson has resulted in a co-authored publication. He successfully initiated research discussions with Drs. Aggarwal, Hackett, and Lubin that led to the plans proposed as Project 2, Project 4, Core A, and Core B. He recruited Dr. Wilson for involvement in this program project. He has secured the commitment of nine potential members of an external advisory panel.

The plan to conduct productivity evaluations at formal annual meetings is appropriate. Indicators to monitor progress and expenditures are not established. Decision to date appear to rely on his personal communications with others. Effective interchange requires a structure commitment to frequent convening of the collaborators as a group to discuss current considerations; however, the absence of a plan for such sessions between annual meetings raises a serious concern.

A process for allocation and reallocation of funding cited as a responsibility within this Core is not documented. A role for the Administrative Core in contributing to the management of resources at the project level is not defined. The program appears to rely largely on effective administration at the level of project and core leaders, with the Administrative Core responsible primarily for interproject communications and reporting.

The issue of potential changes in leadership presents some concerns. A single successor to Dr. F. Barany as the Principal Investigator is not obvious. In the absence of published work in this field, it is not apparent that Dr. Lubin (Project 2) nor Dr. Aggarwal (Project 4) could conduct these projects as proposed in his absence. The plan to include external advisors in internal advisory meetings is

commendable. The application provides letters of commitment from the proposed external advisors, although these letters do not explicitly confirm the expectation to attend an annual meeting. To maximize effectiveness of the annual review, plans are needed regarding preparations for this session and follow-up on subsequent recommendations. The numerous letters of support from other individuals interested in development of the program actually raise concerns about the focus and priorities of the planned studies.

Personnel:

Name: Francis Barany

Degree/Discipline/Date: Ph.D., microbiology, 1981

Role/Percent Effort: Core Leader, five percent

Qualifications/Experience: Dr. Barany is personally involved in the current research collaborations between Project 2 and 4, and Cores A and B. He personally has secured the commitment of potential members of the external advisory panel. Dr. Barany's biographical sketch does not indicate any administrative experience to date beyond leadership of an R01 grant. His recruitment of an experienced administrator as Co-Leader of the Administrative Core shows good judgment.

Assessment in designated role: qualified

Name: Michael J. Bunk

Degree/Discipline/Date: Ph.D., nutritional biochemistry, 1980

Role/Percent Effort: Core Co-leader, 5(YR-01) to 10(YR-02-05)

Qualifications/Experience: Director, Research Resources Management, Strang Cornell Cancer Prevention Center since 1993; previously Assistant Program Director, Clinical Nutrition Research Unit (1987-89), Director of Foundation Relations (1989-91), and Senior Grants Management Specialist (1991-93) at Memorial Sloan-Kettering. Dr. Bunk has served for one year, at an affiliate (Strang) of the applicant organization, in a title very similar to the proposed role. The application, however, does not describe his current duties nor the relevance of this experience to his proposed role. He has a total of six years of potentially relevant prior administrative experience in three different programs outside the applicant organization. The current application and the collaborations developed to date demonstrate administrative skill, but Dr. Bunk's specific contribution to date to these preparations to evaluate the percent effort requested.

Assessment in designated role: qualified.

Budget: A number of administrative systems will need to be developed and established immediately in Year -01. In the absence of evidence that the administrative responsibilities will increase dramatically from the Year 02, it is recommended that the Program Coordinator's effort should remain at 30 percent during this time. The remaining budget is appropriate as requested.

Assessment: Level of merit; good to acceptable.

Women and Minorities in Study Population: All of the proposed clinical materials will come from existing tissue banks from which individual patient identification cannot be made. Therefore this proposal is considered exempt from Women and Minority considerations.

## BUDGET

Project 1 (Dr. Francis Barany, Cornell University Medical College

BUDGET CATEGORIES	Requested	Recommended
THIRD PARTY COSTS		
Direct	71,545	71,545
Indirect	28,959	28,959
TOTAL	100,504	100,504

Project 1 (Dr. Francis Barany, Cornell University Medical College  
(The Children's Hospital))

BUDGET CATEGORIES	Requested	Recommended
PERSONNEL	54,450	54,450
EQUIPMENT	2,595	2,595
SUPPLIES	10,300	10,300
TRAVEL-Domestic	1,200	1,200
THIRD PARTY COSTS		
Indirect Costs		
42 percent	28,959	28,959
OTHER EXPENSES	3,000	3,000
TOTAL	100,504	100,504

Project 2

BUDGET CATEGORIES	Requested	Recommended
PERSONNEL	84,860	84,860
SUPPLIES	14,500	14,500
TRAVEL-Domestic	1,200	1,200
OTHER EXPENSES	3,000	3,000
TOTAL	103,560	103,560

Project 3 (Dr. Francis Barany, Cornell University Medical College

BUDGET CATEGORIES	Requested	Recommended
THIRD PARTY COSTS		
Direct	131,170	131,170
Indirect	60,888	60,888
TOTAL	192,058	192,058

## Project 3 (Dr. Donald Bergstrom, Perdue University)

BUDGET CATEGORIES	Requested	Recommended
PERSONNEL	44,305	44,305
SUPPLIES	13,000	13,000
TRAVEL-Domestic	1,200	1,200
THIRD PARTY COSTS		
Indirect Costs		
51 percent	33,663	33,663
OTHER EXPENSES	7,500	7,500
TOTAL	99,668	99,668

## Project 3 (Dr. Robert P. Hammer, Louisiana State University)

BUDGET CATEGORIES	Requested	Recommended
PERSONNEL	40,800	40,800
EQUIPMENT	4,665	4,665
SUPPLIES	14,000	14,000
TRAVEL-Domestic	1,200	1,200
THIRD PARTY COSTS		
Indirect Costs		
45 percent	27,225	27,225
OTHER EXPENSES	4,500	4,500
TOTAL	92,390	92,390

## Project 4 (Dr. Francis Barany, Cornell University Medical College)

BUDGET CATEGORIES	Requested	Recommended
PERSONNEL	37,847	37,847
SUPPLIES	10,500	10,500
TRAVEL-Domestic	1,000	1,000
THIRD PARTY COSTS		
Direct	60,918	60,918
Indirect	40,815	40,815
OTHER EXPENSES	3,000	3,000
TOTAL	154,080	154,080



Project 4 (Dr. Aneel Aggarwal, College of Physicians and Surgeons  
of Columbia University)

BUDGET CATEGORIES	Requested	Recommended
PERSONNEL	45,918	45,918
SUPPLIES	10,500	10,500
TRAVEL-Domestic	1,500	1,500
THIRD PARTY COSTS	40,815	40,815
OTHER EXPENSES	3,000	3,000
TOTAL	101,733	101,733

## Project 5

BUDGET CATEGORIES	Requested	Recommended
PERSONNEL	64,069	64,069
SUPPLIES	15,500	15,500
TRAVEL-Domestic	1,200	1,200
THIRD PARTY COSTS		
Indirect Costs	85,769	85,769
40 percent Direct	34,308	34,308
OTHER EXPENSES	5,000	5,000
TOTAL	120,077	120,077

## Core A

BUDGET CATEGORIES	Requested	Recommended
PERSONNEL	70,488	70,488
EQUIPMENT	15,000	15,000
SUPPLIES	16,500	11,500
TRAVEL-Domestic	1,200	1,200
OTHER EXPENSES	2,500	0
TOTAL	105,688	98,188

## Core B

BUDGET CATEGORIES	Requested	Recommended
PERSONNEL	60,638	60,638
EQUIPMENT	80,100	80,100
SUPPLIES	11,000	11,000
OTHER EXPENSES	5,000	5,000
TOTAL	156,738	156,738

Core C

BUDGET CATEGORIES-	Requested	Recommended
PERSONNEL	22,836	22,836
TRAVEL-Domestic	9,000	9,000
OTHER EXPENSES	5,500	5,500
TOTAL	38,336	38,336

## SUMMARY RECOMMENDED BUDGET\*

BUDGET CATEGORIES	01 Year	02 Year	03 Year	04 Year	05 Year
Personnel	276,669	343,803	343,803	343,803	343,803
Consultant Costs	1,000	1,000	1,000	1,000	1,000
Equipment	95,100	6,000	76,000	6,000	6,000
Supplies	47,500	43,500	43,500	43,500	43,500
Travel (Domestic)	12,400	12,400	12,400	12,400	12,400
Third Party Costs					
Direct Costs	349,402	350,142	350,142	350,142	350,142
Indirect Costs	164,970	164,970	164,970	164,970	164,970
Other Expenses	16,500	16,500	16,500	16,500	16,500
TOTAL	963,541	938,315	1,008,315	938,315	938,315

\*Appropriate escalation factors to be added at the time of an award.

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